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Photoelectric Measurements of the Luminous Efficiency of Daylight

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(Received 10 March, 1936)

INTRODUCTION—COMPARISON OF VISUAL AND PHOTOELECTRIC SCALES

In the course of numerous measurements of daylight which we have made from time to time with certain stable photoelectric cells (Poole and Atkins, 1935), we have compared the values thus obtained with simultaneous measurements of the total radiation, as recorded by a thermopile. Separate determinations were made for sun and sky by screening off the former by means of a small object held at some distance from the cell, and from the combined results the luminous efficiencies of the radiation from sun and sky on various occasions were found on the photoelectric scale. Some of these results have already been published (Atkins and Poole, 1936).

It might be objected that the photoelectric scale used differs so much from the visual that these figures are misleading. Criticism might, in particular, be directed against the result obtained that the radiation from a clear blue sky generally has a higher luminous efficiency than sunlight, though the weakness of sky light in infra-red explains this. We have accordingly gone into the matter rather more fully, paying especial attention to the question as to the possible overrating of sky light by the selenium cell. It will be seen that, as a result of this further consideration, the general correctness of the previous results has been confirmed.

Many of our measurements of luminous efficiency have been obtained with a Weston selenium rectifier cell mounted under an opal-flashed glass (opal) diffusing surface. This was standardized against artificial "mean noon sunlight" obtained in accordance with the specification of the Seventh International Congress of Photography. This illumination closely resembles natural sunlight in its spectral distribution, except in the violet and ultra-violet, where the artificial source is weak. As this region

is relatively unimportant with a selenium cell exposed to the whole visible spectrum, we can make no large error in assuming that the cell when standardized by the artificial sunlight will give a value for natural sunlight close to that to be found by visual estimation, even though the spectral sensitivity curves of the cell and the eye differ somewhat. We must, however, consider how far we can trust the cell for measurement of sky light.

Fig. 1 shows the spectral response of the cell, mounted under opal, for a natural mean noon sunlight spectrum, each ordinate being obtained by multiplying the equal-energy sensitivity of the bare cell by the relative transmission of the opal and by the relative intensity of sunlight for the given wave-length. The figure also shows the curve for the International Relative Visibility Factor, giving the spectral sensitivity of the average eye. For the eye we have taken the results given in the Smithsonian Physical Tables (1933); for the methods of determination reference may be made to Coblenz and Emerson (1917), also Gibson and Tyndall (1923). This relationship has also been plotted, not for an equal energy spectrum, but for mean noon sunlight. Doing this produces but little change in its form, as the source is of fairly even intensity in the region of the eye's maximum sensitivity. It will be seen that the maxima of the curves for the eye and the selenium cell are not very far apart, but that, as a result of its rather less selective sensitivity, the cell is more sensitive than the eye to both ends of the visible spectrum. The result of this is seen if we standardize the cell (*a*) against a standard filament lamp at a colour temperature of 2360° K, and (*b*) against the artificial "mean noon sunlight" obtained by filtering the light of the same lamp with the liquid filters prepared in accordance with the specification of the Congress. Using the accepted figure 13.52 for the visually determined percentage transmission of the filters we find that, as compared with the eye, the cell is about 1.15 times as sensitive to the 2360° K radiation as it is to the artificial sunlight. Here the richness of the low-temperature source in red has more than compensated for its deficiency in blue, so that the cell rates the "red" source too highly. We might expect from this that it would underrate instead of overrating sky light, but the shapes of the spectral energy distribution curves must be considered, and, as will be seen, this expectation is apparently not justified.

Fig. 1 also shows the response of the cell when mounted under a special green gelatine filter prepared by Dr. L. A. Jones* of the Kodak Labora-

* Liquid filters have been prepared by Preston and McDermott (1934) which reduce colour sensitivities of certain thin-film emission cells to a close approximation to that of the eye.

tories in 1934, for samples of which we are indebted to Dr. C. E. K. Mees and to Dr. Jones. These filters are designed to reduce the response of the Weston selenium cell to close accord with that of the eye, when one is

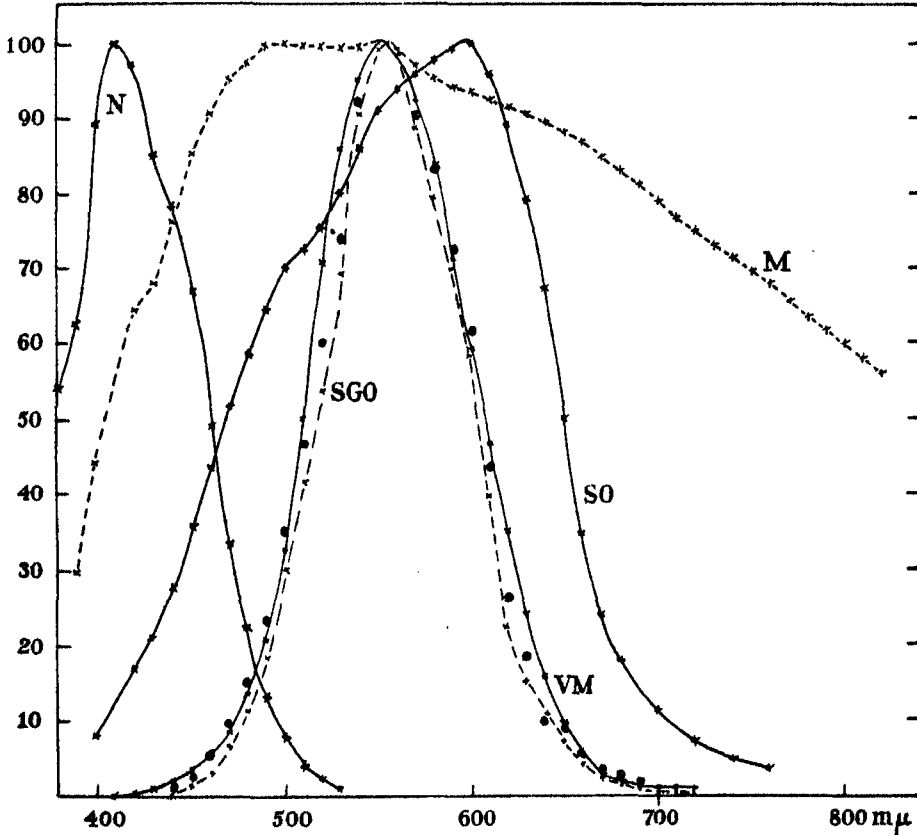


FIG. 1—Curve M shows the distribution of energy in mean noon sunlight, as a percentage of the maximum, and all sensitivities relate to this spectral energy distribution. N denotes sodium vacuum cell under opal. SO denotes selenium cell under opal. SG denotes selenium cell under green filter; as this lies so close to the VM and SGO curves it has been omitted, but it is denoted by the crossed circles not on a curve; SGO shows the foregoing under opal, taking into account that the filter is now traversed by oblique light. VM denotes the international relative visibility factor of the eye, as for mean noon sunlight. The curves intersect the axis at the red end further on.

mounted in front of the bare cell, and used for light incident normally. The accord is so close that the curve (SG) for the combination is represented only by crossed circles which lie on either side of the curve for the eye (VM). As, however, we need to measure the diffuse light from the

sky as well as the direct light from the sun at various altitudes, we must use the opal as a diffusing surface, and, in order that the filter should have comparable effects on direct and diffuse incident light, it must be placed behind the opal, where the light is always diffuse.

The use of a filter in diffuse light increases its effective thickness. From some tests on approximately non-selective filters we have concluded that a 1 mm filter is about equivalent to a similar one 1.15 mm thick if it is used in diffuse light. As a result the selective absorption of the filter is enhanced. Moreover, the glass under the opal-flashed layer has a greenish tinge when viewed on edge, and tests showed that the combination absorbs appreciably towards the blue end of the visible spectrum. Accordingly, as will be seen from curve SGO, fig. 1, the cell with filter under opal, is somewhat overcorrected, being particularly insensitive in the blue and violet. This combination, therefore, when standardized in sunlight (natural or artificial), would almost certainly underrate blue sky light in comparison with the eye. We should expect the visual estimate of sky light to lie between those found using the cell and opal with and without the green filter.

TABLE 1—Apparent Transmission of Kodak Special Green Filter Mounted under Opal, as Measured by Weston Selenium Cell for Various Types of Illumination

Source of illumination	% transmission
Filament lamp at 2360° K	20.16
Artificial "mean noon sunlight"	22.00
Natural sunlight	20.52
Mixed daylight	20.25
Clear blue sky light	19.08

These filters being of the gelatine type could not be assumed to possess sufficient permanence to justify frequent use in bright sunlight. We therefore only used the green filter on a few occasions, making both relative determinations of the percentage transmission of the filter as mounted under opal for various types of illumination (*see* Table I) and some measurements of the intensity of sunlight and sky light (*see* Table II) as recorded by the cell with and without the filter respectively, standardization being effected by means of artificial "mean noon sunlight" in each case.

It will be seen that in comparison with the uncertainties which always occur in the measurement of daylight, the differences are scarcely significant although the variation in colour covered by the illuminants is so large.

As might be expected from fig. 1, the green filter shows a reduced transmission in sky light. We should expect the values for natural and artificial sunlight to agree, rather than those for natural sunlight and lamp light, but the discrepancies are not very serious, and it is clear that the ratio of sky light to sunlight should not vary by more than 10% according as the cell is used with or without the green filter. As the cell with filter almost certainly underrates the light of a clear blue sky this seems to be good evidence that the cell without filter is not seriously overrating it.

As a further test, we used the cell and filter standardized against the artificial sunlight source to measure the intensities and luminous efficiencies of natural sunlight and blue sky light on two dates with different lighting conditions; almost simultaneous readings were obtained with the cell without filter, and with the solarimeter. Though the selenium cell used remained constant to within at least 0.5% over its period of use, we are of the opinion that there may be an error in the luminous efficiency determinations of perhaps as much as 2% owing to the different conditions of standardization involved when the additional filters were used; an uncertainty arises as to the scattered light from black surfaces, so that unless conditions are identical a change may have been introduced. The number of diaphragms used is limited by the large size of the source and its relatively feeble emission which rendered 40 cm distance the optimum. When we say that the cell remained constant, this refers to constancy of sensitivity under the conditions of the standardization. Temperature effects may, we believe, cause variable errors, though the use of a low resistance measuring instrument greatly reduces these, as we have shown elsewhere (Poole and Atkins, 1933, 1934). Also, on one occasion, a Burt sodium vacuum cell was compared, using a constant for the latter which accords with the selenium cell in average bright mixed daylight. The results are given in a subsequent section.

LUMINOUS EFFICIENCY AND ITS DETERMINATION

The rate of flow of energy, Φ , may be expressed in ergs per second, or in watts. The luminous flux, F , is the rate of flow of radiation measured according to its ability to produce visual sensation. The unit is the lumen, the flux emitted in unit solid angle (steradian) by a source of unit candle power. The luminous efficiency = F/Φ expressed in lumens/watt. Now 1 kilolux on a square metre is a flux of 1000 lumens, or 0.1 lumen per sq cm, and our radiation intensity W is most conveniently expressed in milliwatts per sq cm. Expressing illumination I , in kilolux, $F/\Phi = 100 I/W$; the values are tabulated as lumens per watt.

For measuring radiant energy throughout the whole spectrum, we used a Moll large surface thermopile, made by Messrs. P. J. Kipp. This when standardized by the makers gave an E.M.F. of 5.3×10^{-8} volt for $1 \text{ erg sec}^{-1} \text{ cm}^{-2}$ on open circuit, namely, 1 microwatt/sq cm corresponds to 0.53 microvolts. These figures refer to the instrument when used uncovered. It is provided with a plane disc of colourless glass 3.25 mm thick, which fits into the tube. This will cut off radiation shorter than about 0.32μ and longer than about 3.5μ , and reflexion losses reduce the transmission in and near the visible spectrum to about 92%, with a somewhat higher value in the far infra-red. We found that this glass transmitted 85.7% of the radiation from a sub-standard tungsten filament vacuum lamp run at 2360° K .

In measuring radiant energy within the limits transmitted by glass, we used a Moll solarimeter which has two hemispheres of glass, 50 and 30 mm diameter respectively, mounted over the thermopile. The makers' standardization shows an E.M.F. of 7.77 millivolts per gram calorie $\text{cm}^{-2} \text{ min}^{-1}$ on open circuit, or 0.112 microvolt for 1 microwatt/sq cm. The method of standardization adopted by Messrs. Kipp is to compare these solarimeters with another one which has been carefully calibrated against an Ångström pyrheliometer; it is re-calibrated from time to time.

Except where otherwise stated, all our radiometric measurements in the open were made with the solarimeter, which, being suitable for measuring the vertical radiation from a complete hemisphere, and having been standardized in the open by the makers on the Ångström scale, formed a suitable standard. A comparison of the two instruments when exposed to the radiation from an arc showed agreement to within about 1%, the makers' constants being used. In this case the large surface thermopile, which was used bare, was affected by the complete range of radiation, whereas nearly all over 3.5μ and all under 0.32μ was stopped by the glasses of the solarimeter. When the instruments were compared in sunlight (by comparing the vertical sunlight found from the difference between the shaded and unshaded readings of the solarimeter, with that calculated from a measurement of the perpendicular sunlight with the large surface thermopile) the effect of both the long and the short wave radiation was less important, so that the two instruments no longer agreed exactly, the mean value of two such comparisons, carried out in June with solar altitudes 50° and over, being 0.93 for the ratio thermopile/solarimeter. As we have adopted the solarimeter as a standard, we have multiplied the constant supplied by the makers for the surface thermopile by 1.08 in finding the four values of the luminous efficiency of sunlight obtained with the thermopile.

One very important point must be noted as regards the use of these instruments, namely that they do not attain equilibrium instantaneously as does the photoelectric cell. This is very noticeable in obtaining the ratio, β , of sun plus sky to sky, with the solarimeter; for unless one waits an appreciable time, variable according to the conditions, the shaded reading will appear too high, since the instrument, including the double glass hemisphere, takes an appreciable time to cool. (One cannot make a determination of β with the bare thermopile, since the blackened surface is placed 2.40 cm behind the outer rim of the tube, which accordingly shades much low-angle radiation.) We had therefore to use our judgment in each individual measurement as to when a reasonable time had elapsed for the attainment of equilibrium, as distinct from alterations in the atmospheric conditions affecting radiation and cooling. This introduces an element of uncertainty which, however, seems unavoidable, though we were always on the watch to render it as small as possible.

As regards the accuracy of radiation measurements (which concerns our own determinations of luminous efficiency described later), according to Abbot, Fowle, and Aldrich (1913), the values obtained with Ångström pyrheliometers are on the average 3.9% below those obtained with the Abbot silver-disk pyrheliometers. Abbot and Aldrich (1932) state that measurements with their improved water-flow pyrheliometer lead to a revision of the Smithsonian standard scale of solar radiation, so that the value formerly given by them for the solar constant, $1.940 \text{ cal cm}^{-2} \text{ min}^{-1}$, would become as corrected 1.893, than which the old value is 2.5% greater. Their most recent paper gives the correction as 2.3% (1934). They quote Tingwaldt of Davos as giving a positive correction of 1.9% to the Ångström scale and a correction of -1.8% to the Smithsonian scale of 1913. This agrees in direction with that given by Abbot and Aldrich, but their own correction is greater. Thus the reduction in the Smithsonian scale of 2.3% should lessen the discrepancy with the Ångström scale, leaving the difference 1.6% or 1.9% according to Tingwaldt. We have not attempted to introduce this correction into our results, which are on the Ångström scale, but have contented ourselves with stating that this scale is probably about 1.6% low, which will, of course, raise our values of luminous efficiency as compared with American work on the other scale; thus Kimball's value for luminous efficiency, recalculated in lumens per watt, becomes 105.1, on the Ångström scale, instead of 103.4.

In converting the constants of the instruments, we have taken it that one joule = 0.2391 gm cal at 15° C, or $4.183 \text{ j} = 1 \text{ gm cal}$. Accordingly $1.00 \text{ millivolts} = 8.97 \text{ milliwatts cm}^{-2}$ for the conversion of open circuit

voltage readings with solarimeter No. 296. It may be noted that radiation at the rate of $1.00 \text{ gm cal cm}^{-2} \text{ min}^{-1}$, is equal to $69.7 \text{ milliwatts cm}^{-2}$ and in general we have adopted the milliwatt for the expression of our results, though in some cases the calorie is more convenient, and was used.

MEASUREMENTS OF THE LUMINOUS EFFICIENCY OF DAYLIGHT

Kimball made determinations of the relation between visual measurements of daylight and radiation. These were probably made by comparison with the Macbeth or similar illuminometer and the figure he gives, 6700 foot candles = $1.00 \text{ cal cm}^{-2} \text{ min}^{-1}$, is stated to hold within $\pm 5\%$ on clear days. Our own variations are much wider, which seems natural in view of the extremes of the meteorological conditions which we selected for examination; but it may be seen in Table III that Kimball's value is very close to ours for sun alone on 1 May and 12 October and for sun and sky on the afternoons of 22 February and 10 October. No values for sun and sky separately have been found by us in the literature.

We are considering first observations that were actually made last, since the validity of the earlier ones depends to a considerable extent upon these, as summarized in Table II.

It is evident that the cell without filter overrated sunlight by about 13% and sky light by 19.5% as compared with the cell with green filter on 12 September, *i.e.*, it overrated skylight by 6.5% relative to its own rating of sunlight. On the other hand, on 21 October its rating of sunlight was 0.7%, and that of skylight 14% lower than the corresponding values for the cell with filter. The difference is typical of anomalies that are somewhat common in daylight measurements. It is probably entirely due to the fact that on the first occasion the sky was a very clear deep blue while on the second a lighter blue and wisps of cloud contributed to render the illumination considerably nearer to white. Had the filter used faded appreciably a somewhat similar result would have been obtained, but comparison with a duplicate filter exposed only to artificial light or weak daylight for short periods gives no visual indication of any such change.

Taken in conjunction with the curves shown in fig. 1, it is evident that the cell under the filter and opal (which was always used) does not overrate blue sky light on the visual scale, and that the cell without filter does not do so by more than a few per cent. We cannot be equally certain as to the extent to which it may be underrating it, but in view of the large figures that we get for its luminous efficiency underrating seems to be improbable.

We also give in Table II values for the "luminous efficiency" as found

TABLE II

Almost Simultaneous Determinations of Luminous Efficiency of Daylight Using Weston Selenium Cell, Without and With Kodak Special Green Filter Under Opal, and Burt Sodium Cell under Opal. Illumination about 71 and 55 kilolux on Two Dates, Respectively, for Sun and Sky, with Selenium Cell

Time	Conditions	Cell	No. of sets of obs.*	Lumens per watt		
				Sun and sky	Sun	Sky
12 September, 1935, 2.25-2.45	Very clear sky, wind S.E., white clouds in N.E.	Selenium	4	112.8†	113.1	114.3
		Selenium with green filter	5	97.8	98.6	91.9
21 October, 11.10-12.0	Clear sky, wind N.W., at start, later numerous wispy clouds, cloudlets near sun, low clouds in N.	Selenium	5	107.8	99.5	130.6
		Selenium with filter	6	107.0	100.2	149.1
		Sodium	3	104.5	72.2	242.0

* The values given are the means for the number of sets of observations indicated; but each observation entered up in a set was actually the mean of a number of galvanometer readings, how many were taken in each case depending upon the variation in the illumination at the time.

† An additional observation for sun and sky slightly lowered the average.

with a Burt sodium vacuum cell which we have used for a number of years for the continuous recording of daylight. This, as will be seen from the curve plotted in fig. 1, is only sensitive to the blue, violet, and ultra-violet. We have standardized it by comparison in bright mixed daylight, with a potassium vacuum cell which had been standardized against the carbon arc. This "potassium arc scale", which we have always used, fortunately agrees almost exactly in mixed daylight with the "selenium mean noon sunlight scale" which we have now adopted. Accordingly, in such illumination the Burt cell gives a reading and hence a luminous efficiency, very close to that found with the selenium cell. It is found that over quite wide ranges of mixed daylight the discrepancy between the two cells is seldom serious, but the Burt cell, as is natural, underestimates pure sunlight and seriously overestimates the light of a clear blue sky. Thus the "luminous efficiencies" found by it for such illuminations vary very considerably from the visual scale. The chief purpose to be served by their determination is that of enabling estimates of the total energies of radiation to be made from the numerous readings taken, under varying light conditions, with this cell during the past few years. Numerous determinations of luminous efficiency made with the Burt and selenium cells are shown in fig. 2.

Table III gives a selection of the results obtained with the selenium cell at different seasons with illuminations varying from 696 lux, from the sky just after sunset, to 84·8 kilolux. In spite of the fact that it is over-rated by the cell, winter sunlight gives relatively low values for the luminous efficiency, minimum in Table III being 47·4 and maximum 116·0, the corresponding values for sky light being 111·9 and 136·4 and for mixed daylight 81·1 and 117·3 lumens/watt. The average for sun and sky is 107·8, which is close to the value calculated from Kimball's results, namely, $105·1 \pm 5\%$.

Other values for luminous efficiency obtained with the selenium cell are shown in fig. 2. These extend the limits given in Table III down to 35·5 lumens/watt for winter sunlight and to 48·5 for a dull grey winter sky and up to 120 for summer sunlight and 158 for light blue sky. The values for mixed light are as a rule round about the average given by Kimball, as indicated by a line across fig. 2. This figure also shows a number of determinations of luminous efficiency obtained from measurements of illumination with the Burt sodium cell. In general the determinations are in good accord with the visual for mixed daylight. The minimum value 43·8 lumens/watt for a very dull grey sky is nevertheless above that of most artificial sources. A very clear blue sky in May gave 229 lumens/watt at Plymouth and in June as much as 337 lumens/watt at Dublin.

In a previous paper (Atkins and Poole, 1933) we made a rough comparison between the radiation, as measured in London for the year 1930, and the illumination integral, expressed in kilolux hours, as measured at Plymouth with the sodium cell for the same year. It was found that one

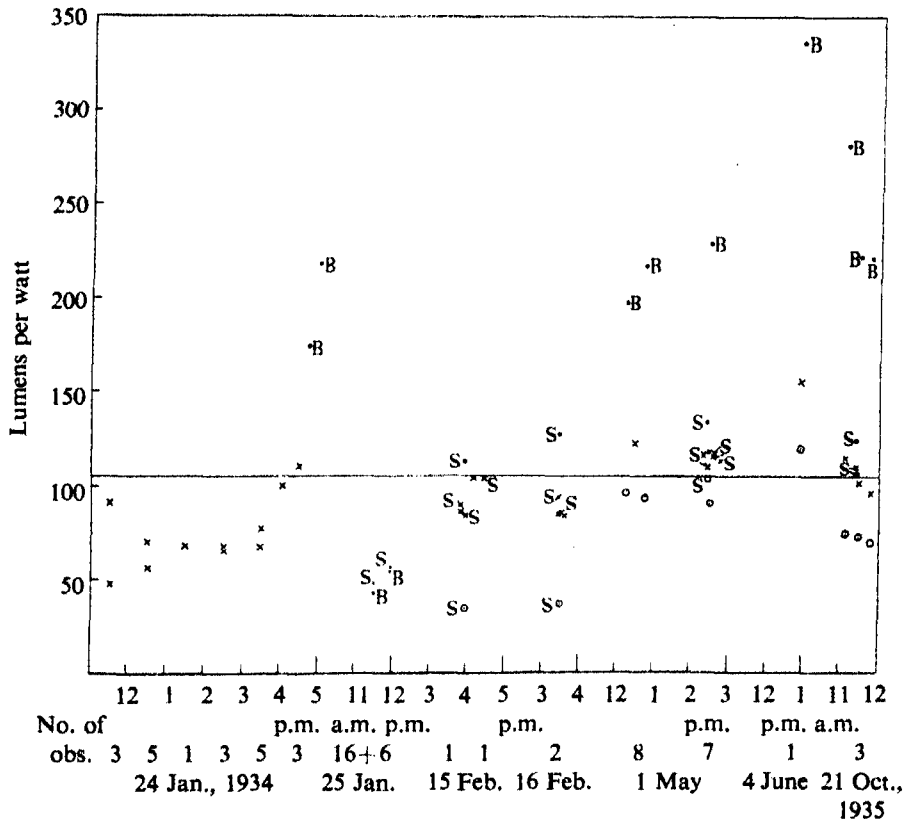


FIG. 2.—The ordinates denote luminous efficiency, in lumens/watt. The abscissae show time on various dates, as marked. The row of figures, below the hour figures, gives the number of observations with the Burt sodium cell, the mean results of which are given; single observations are plotted for the selenium cell. For the sodium cell luminous efficiency results for light from sun and sky, as received on a horizontal surface, are shown by crosses, to which S is added if selenium cell was used instead. Circles with dots give the corresponding results for the sun, S being added as before. For sky light dots marked B (Burt) or S are shown. The line at 105 lumens/watt shows Kimball's visual determinations for sun and clear sky.

kilolux hour was equivalent to 0.404 gm cal. Direct comparisons made with the same cell and the Moll solarimeter at Plymouth give 0.375–0.493 for sky alone, when blue, and 1.97 for a very dull grey sky; for sun alone 0.892–0.950 and for sun and sky 0.78–1.79 gm cal per kilolux hour.

TABLE III

Relation* between Vertical Illumination as Measured with a Selenium Rectifier Cell Standardized in Artificial Mean Noon Sunlight and Energy as Measured with a Moll Solarimeter.

Date	G.M.T.	Nature of light	Sun, sky, or total		Lumens per watt		
			Kilo-lux	mw cm ⁻²	Sun and sky	Sun	Sky
22 February, 1934	4.0	Very clear blue sky, no clouds, wind S.S.E., $\alpha = 14.6^\circ$	Sun 19.08	23.4	81.5	58.2	124.0
	4.31	Sun $\alpha = 10.6^\circ$, $\beta = 2.27$ by solarimeter and $\alpha = 14.6^\circ$ by cell	12.16	15.0	81.1	47.4	124.0
	5.40	Sun red, touching horizon	0.884	0.85	104.0	—	—
	5.43†	Sun just gone	0.696	0.53	—	—	131.0
1 May, 1934	2.23	Clear light blue sky, wind S.S.E., light	84.80	72.5	117.0	—	—
	2.27	Sun $\alpha = 45^\circ$, $\beta = 4.67$ by solarimeter and 3.87 by cell	79.46	72.1	110.2	103.7	134.0
	2.36	Clear light blue sky	81.78	70.2	116.4	—	—
	2.38		81.43	69.7	116.8	—	—
	2.40		81.26	69.2	117.3	—	—
	2.49		77.50	68.3	113.4	—	—
12 September, 1935	2.16	Sky exceptionally clear, white clouds in N.E., wind S.E., light	74.10	63.5	116.8	116.0	119.4
	2.20		71.76	64.4	113.3	111.2	111.9
10 October, 1935	11.32	Blue sky with clouds in W. and N.	64.04	58.1	110.3	—	—
	11.40		65.11	60.1	108.4	—	—
	11.45		65.68	60.8	108.0	—	—
	11.53		66.58	60.8	109.6	—	—
12 October, 1935	11.27	Clear sky, wind N.W.	55.18	50.1	110.2	106.2	124.8
	11.32		51.20	50.3	101.6	92.7	136.4
Averages			—	—	107.8	90.8	125.7
†Average sun and sky, Kimball $\pm 5\%$, visual observation			—	—	105.1	—	—

* The results given here are only a selection from more numerous determinations; some of the others are plotted in fig. 2, and the maximum and minimum values are quoted in the text.

† Reduced from the Smithsonian to the Ångström scale of radiation as explained in text, since our own radiometer was calibrated on the latter.

From Kimball's figures one obtains about 0.83 gm cal on the visual scale. This quantity is useful in applying the large number of observations, obtained during the last six years, to calculations concerning the warming up of the sea owing to the absorption of visible light; the effect is quite appreciable far beyond the range of penetration of even the visible red, and a rough estimate has already shown a tolerably good agreement between the observed temperature changes, below the thermocline, and the heat thus supplied.

THE LUMINOUS EFFICIENCY OF SOME ARTIFICIAL AND NATURAL SOURCES

Values are given in the 'International Critical Tables, 1929,' vol. 5, p. 437, and by Fowle (1933), showing the efficiency of various sources of artificial light. The "Luminous Equivalent of Radiation of Maximum Visibility" (taken here for $\lambda = 0.556 \mu$), obviously the upper limit of efficiency, is 668 lumens/watt, or 1 lumen = 1.496 mw. Fowle quotes this value from Hyde, Forsythe, and Cady, but the earlier value in the I.C.T. is 1.61 mw/lumen, giving 621 lumens/watt. It is there stated that the figure given depends to some extent upon the conditions of observation (illumination size of field, type of photometer), and that there is no general agreement as to what these conditions should be. In using the compilations mentioned, one must distinguish carefully between the "Luminous Efficiency", namely, "Luminous Flux/Radiated Power", and the "Efficiency of Source", *i.e.*, "Luminous Flux/Input Power". In the tables these are given under E and E_s (this, of course, has nothing to do with Aurén's E_s unit mentioned in our previous paper), respectively, but some tables give the "Relative Luminous Efficiency", E_r , which is the luminous efficiency relative to that of radiation of maximum visibility. In the literature it is not always easy to ascertain which is intended. E and E_s do not differ very greatly for filament lamps where most of the input is radiated, but they do so for flames and arcs.

Thus, the British standard candle burning 120 grains (7.75 gm) per hour has an input of about 81 watts giving E_s as about 0.0125 lumens/watt. The value of E_r given for it in the I.C.T., *i.e.*, 0.24%, corresponds to a luminous efficiency, E , as high as 1.5 lumens/watt, the large discrepancy being due to the fact that only some 1% of the input energy is radiated, the balance being lost by convection.

Our own measurements with the selenium cell on the open carbon arc, in a position in which the positive carbon is quite unscreened, give for the luminous efficiency about 28 lumens/watt, the efficiency of the source as

derived from the input power being estimated as about 14 lumens/watt. Table 356 (Fowle, 1933) quotes Ives as follows on the basis of 1 lumen = 1.59 mw:—open gas flame burner 0.22 lumens/watt; incandescent gas, high pressure, 2.0 lumens/watt; carbon arc open, 9.6 amp, 11.8 lumens/watt; tungsten vacuum filament lamp 8, gas filled 500 watt, 15 lumens/watt; quartz mercury arc 42 and yellow flame open carbon arc 44.7 lumens/watt. These figures are based on the input in watts.

All our own values are for E, the luminous efficiency of radiation; our results for the selenium cell under opal, but without selective filter, run from 35.5 (low sun) to 120 (high sun) and 48.5 (grey sky) to 158 (light blue sky). These are far higher than any artificial sources that can be used as standards.

It is of much interest to compare the luminous efficiency of sun and sky with that of light of animal origin. The maximum efficiency possible is 668 lumens/watt as previously mentioned. According to Abbot and Fowle (1908), the Cuban firefly, *Pyrophorus noctilucus*—and presumably other similar luminous animals—are far more efficient than any other source. They report the insect named as giving light as less than 1/10,000 part of the expenditure of energy required for an equal amount of light from the candle.

This would seem to imply that the overall efficiency of the firefly as a source was 10,000 times that of the candle, *i.e.*, 125 lumens/watt, an extraordinarily high value when we consider that much of the input energy of the firefly must be used up in other ways, in evaporation, for instance. Should we, however, take the figures as referring to the luminous efficiencies, the total energy radiated being measured for each source, we get the impossible value 15,000 lumens/watt for the firefly—about 22 times that of light of maximum visibility. It is evident that the measurements of the light, the input, and the radiated energy, for a source such as the firefly, are difficult and uncertain.

According to Coblenz (1912), the temperature of the firefly is slightly lower than that of the air and the luminous segments are slightly warmer than the non-luminous, but no definite change could be detected during a flash. Harvey (1919, 1924, 1927) has shown that in such animals no carbon dioxide is produced in the emission of light. The luciferin of the crustacean *Cypridina hilgendorffii*, however, when oxidized to oxyluciferin, has a total efficiency of the luminous process, *viz.*, energy in luminescence/heat of reaction, of about 1%. The maximum of its spectrum is at 0.48 μ . Luminescence is not an invariable accompaniment of luciferin oxidation, more than one molecule must react to produce one quantum.

It is obvious, however, that the ratio of the light flux to the total energy

radiated must be high, since though the light of different luminous organisms may vary through all spectral tints from red to violet, no infra-red is given out. As a rule yellowish, greenish, or blueish hues predominate. A further consideration of these interesting researches would be foreign to the aim of this paper.

We desire to express our thanks to the Government Grant Committee of the Royal Society for a grant for the purchase of photoelectric cells and thermopiles.

[*Note added in proof*, 28 May, 1936—The accuracy of the physical measurements of illumination and radiation is limited in this work by the following considerations:—

- (a) The illumination in the open is rarely constant to within 1% for one minute and may easily vary by 10% or more; the radiation appears to be less variable, but this is due to the lag of the thermopiles and their galvanometers.
- (b) Exposure in the open under varying conditions unavoidably leads to alterations in the sensitivities of the measuring instruments owing to temperature changes.

Care was taken to minimize errors in (a) by taking averages of a number of readings and by two observers making simultaneous measurements. A nearer approach to a simultaneous reading may be obtained when one observer reads the thermopile galvanometer deflexion and makes the final adjustment for the measurement of illumination by bringing the potentiometer-telephone-amplifier null method apparatus to silence, for this can be done with great rapidity. Thus the measurement is effected by the senses of sight and hearing of the same observer and obviously avoids the lag occasioned when one observer gives a verbal signal to the other. This method was only used in comparing different instruments. Errors in (b) were minimized by shielding the instruments from direct sunlight as far as possible and alterations in zero were always checked.]

SUMMARY

Comparisons of the illumination recorded by a Weston selenium rectifier cell under opal flashed glass, standardized by means of artificial "mean noon sunlight", with simultaneous measurements of the total radiation found with a thermopile have given high values for the luminous efficiency of the radiation from a clear blue sky.

To test for possible serious discrepancy between this photoelectric scale and the visual scale when blue sky light was being studied, use was made of a special filter designed by Dr. L. A. Jones to reduce the spectral sensitivity of the Weston cell to the visual scale. Measurements show that the sensitivity curve for the bare cell used under the filter for light incident normally agrees well with the International Relative Visibility Factor.

When used under opal, as is necessary for measurements of diffuse light, the combined filter and opal caused some additional absorption of the blue end of the spectrum. Thus the combination of cell with filter and opal must somewhat underestimate the light of a blue sky. Comparisons of the relative intensity of a tungsten vacuum lamp at 2360° K, sunlight, and sky light with and without the filter gave little difference in the percentage transmission of the filter for these very dissimilar sources, showing that for such sources the excessive sensitivity of the Weston cell to both ends of the spectrum approximately balances, so that no large discrepancy exists between the photoelectric and visual scales. The cell without filter gave somewhat higher readings of blue sky light on the average. Thus it may overestimate this light by a few per cent on the visual scale.

Numerous determinations of the luminous efficiency of daylight were made with this cell without the special filter. The variations under different meteorological conditions were large, pure sunlight ranging from 35.5 to 120 lumens/watt, and sky light from 48.5 for a very dull grey sky to 158 for a clear light blue sky. The luminous efficiency of blue sky light generally appears to be higher than that of sunlight, since the former is relatively very poor in infra-red. For mixed sunlight and sky light values were obtained ranging from 81.1 lumens/watt, on a winter afternoon to 119.4 for a very clear day in early September.

Using the Weston selenium cell with the special Kodak green filter under opal, the sun was one day found to be rather more efficient than the sky, which was a deep blue. With a lighter blue sky and some white clouds the sun gave 100.2, the sky 149.1, and both together 107.0 lumens/watt.

Measurements were also made with a sodium vacuum cell, which is only sensitive to the blue end of the spectrum. When suitably standardized this cell generally agreed well with the selenium cell in mixed daylight, but, naturally, it underrates sunlight and overrates blue sky light. Thus the "luminous efficiencies" found by its aid for sunlight and sky light differ considerably from the true visual values, and are chiefly of use for coordinating a great number of daylight measurements, made with it

over a number of years, with the corresponding values of the total radiation.

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Hearing in Insects: The Nature of the Response of Certain Receptors to Auditory Stimuli

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[PLATES 1 AND 2]

The auditory receptors of the Orthoptera include tympanal organs and hair sensilla. The former have long been recognized as subserving an aural function, but the latter have, in this group, only recently been shown to respond to auditory stimuli (Pumphrey and Rawdon-Smith, 1936).

Wever and Bray (1933), using an amplifier and telephones, have attempted to analyse the nervous response from the tympanal organs of the foreleg of crickets (*Lyogryllus campestris*). Their operational technique may be shortly described. Without previous dissection, a fine wire electrode was introduced into the leg of the experimental animal, the return lead consisting of a pad applied to the animal's body. Owing to the fact that no direct contact was certainly made with the tympanal nerve and that the central ends of the leg nerves were not severed, responses of several kinds were obtained. Of these, an asynchronous* response, described as producing a shushing noise in the telephones, was thought to arise from the tympanal organ; but no certain indication was given that this response was not partly or wholly of tactile origin. This point is of importance since, as we shall show in this paper, a response from certain types of tactile receptor may be readily elicited by an auditory stimulus. Similar criticisms apply to the recent results of Wever (1935) on the tympanic organ of the grasshopper (*Arphea sulphurea*).†

APPARATUS

To Wever and Bray's electrode system we have preferred the use of two fine platinum electrodes both in contact with the active nerve. These

* Throughout this paper we use the term *synchronous* in the sense in which it is employed by Davis (1935), i.e., the occurrence of one impulse or volley of impulses for each cycle of the stimulating wave.

† In this experiment the active electrode was placed "in or near the third thoracic ganglion" (*loc. cit.*, p. 18).

were connected to an amplifier, the first stage of which is modified from the balanced type designed by Ross (1935). By the adoption of the balancing potentiometer described by him, and the avoidance of one earthed input lead, complete freedom from electrostatic pick-up from the loud-speaker or its leads may be readily secured. This point is of importance when, as in our work, the use of an electrically driven tone source is contemplated. The remainder of the amplifier is of the conventional resistance-capacity coupled triode type; the output stage is condenser-coupled to a cathode ray oscillograph. The H.T. supply for the later stages and the gun voltage for the oscillograph are obtained by transformation and rectification of the A.C. mains. No trace of ripple from this source has been detected. For observation of the electrical response a synchronized linear sweep has been employed. This apparatus is similar to that described by one of us elsewhere (Hallpike and Rawdon-Smith, 1934). For stimulating purposes the following apparatus has been used. A heterodyne oscillator is coupled through an amplifier to a loud-speaker. By means of L-section attenuating networks, the intensity of the pure tone derived from this source may be controlled over a range of 140 db in 2 db steps, whilst, by means of the variable capacity tuning of one of the R.F. oscillators, any frequency between 30 and 11,000 c/sec may be readily secured. A more complete description will be found in the references given (Rawdon-Smith, 1935, 1936).

The animal is installed in a sound-proof electrostatically shielded room; all the stimulating and recording apparatus with the exception of the loud-speaker used for stimulating is situated in another room adjacent. In order to localize the stimulus without exaggerating electrostatic escape from the loud-speaker leads, the sound from the latter, an instrument designed to operate into a considerable acoustic load, is led to the animal through a length of earthed flexible metal tubing.

THE ANAL CERCUS OF THE CRICKET (*Gryllus domesticus*) AND COCKROACH (*Periplaneta Americana*)

The structure of these appendages has been described by Sihler (1924), who has shown that they contain sensilla of three types; long, slender sense-hairs, short sense-hairs, and campaniform organs (Sinnesköpfe). On the cercus of the cockroach the short sense-hairs are generally distributed, but the campaniform organs and the long, slender sense-hairs are limited to the dorsal and ventral aspects respectively. Since vaseline applied to the ventral surface abolishes the nervous response to sound stimulation, while on the dorsal surface it has no detectable effect, it

seems certain that the long, slender sense-hairs are the receptors with which we are here concerned, and the very close similarity between the response of the cerci of the cockroach and cricket appears to justify the belief that the same kind of receptor mediates the response in both cases. Each sensillum is innervated by a single afferent fibre, and the cercal nerve is composed principally of these. It runs from the base of the cercus to the last abdominal ganglion and may easily be exposed from the ventral surface of the abdomen and, after section close to the ganglion, placed on the electrodes.

There appears to be no lower limit to the frequency to which this organ will respond. Indeed, it is extremely sensitive to gross movements of the surrounding air. The response to a gentle puff is shown in fig. 1, Plate 1. Little response, however, can be detected above 3000–4000 c/sec. The auditory spectrum of the organ of the cricket was investigated in the following way. The intensity of a sound, initially subliminal, is increased until a just perceptible response is obtained from the nerve; with the apparatus we have been using this corresponds to an electrical input of perhaps 3–5 microvolts. The procedure is then repeated for various frequencies, and the results thus obtained, together with those secured with the same apparatus for a normal human threshold,* are compared in fig. 2. It is clear from this curve that sensitivity of this organ to low frequencies is much greater than that of the human ear. Indeed, as may be deduced, a marked response may be obtained to a stimulus completely inaudible to man.

Physiologically the response to sound stimuli resembles in many respects that obtained from the mammalian cochlear nerve. At the lower end of the frequency spectrum the response is almost completely synchronized with the stimulus frequency, fig. 3, Plate 1. This phenomenon persists for a considerable time during a sustained stimulus. At higher frequencies, synchrony of the response is only partial and persists only for the first fraction of a second of stimulation (*see* fig. 4, Plate 1, and figs. 9, 10, Plate 2). Similar phenomena may be demonstrated in the response of the VIIIth nerve of the cat between 1 and 4 k/c/sec (Davis, 1935, etc.).

Over the lower range of frequencies, a curious effect may be remarked. Immediately after the onset of a stimulus, the organ appears sometimes to respond to both phases of the stimulating wave. Thus, a stimulus of 400 c/sec may give rise to a response consisting of a short burst of waves

* The normality of this threshold is adequately attested by the results of many measurements with a Western Electric 2-B Audiometer.

at a frequency of 800/sec (fig. 5, Plate 1), after which the normal 1:1 relation obtains, giving but one response per stimulus cycle. (This effect we have termed *frequency-doubling*.)

At frequencies higher than these, the opposite effect may be noticed; thus, a 600 c/sec tone will frequently elicit only one response crest per two cycles of the stimulus; this is not always obvious from the photographic

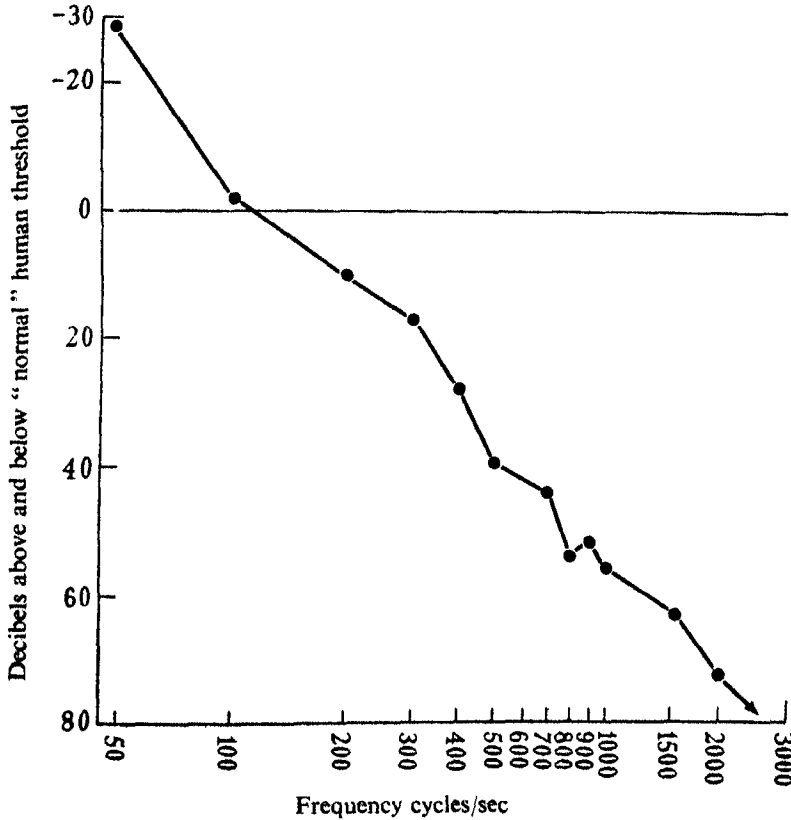


FIG. 2—Graph showing sensitivity of cercus of *cricket* compared with "normal" human ear. Negative sign on the decibel scale indicates sensitivity greater than human.

records obtained, owing to the large asynchronous component often manifested (see, however, figs. 11, 12, Plate 2), but is readily noticeable if the nervous response is made audible by means of a loud-speaker. The drop in pitch of the resultant sound to a note one octave (or at some frequencies two octaves) below the stimulus frequency is easily observed. These effects we have termed *frequency-halving*, *quartering*, etc., as the case may be.

For somewhat lower stimulus frequencies, in the neighbourhood of 400 c/sec, moreover, a reduction of stimulus intensity of perhaps 10 db may often result in a substitution of the 1:2 response relationship for the normal 1:1 discharge. That is to say, the higher intensity 400 c/sec note produces 400 response waves per second, but at the lowered intensity the frequency of the latter falls to 200 per second. A rise in the relative amplitude of the asynchronous component accompanies this effect.

There seems little doubt that these effects (frequency-halving, etc.) represent examples of the phenomenon of "alternation", though of a type somewhat different from that usually associated with the mammalian auditory response (Derbyshire and Davis, 1935). It is now generally accepted that the reproduction of high stimulus frequencies in the VIIIth nerve involves that each fibre responds, as in our case, to every other stimulus wave. Owing, however, to the fact that the electrical response in different fibre groups is 180° out of phase, the response from the whole nerve still reproduces the frequency of the stimulus. In our own case this is not always so, and we have a clearer demonstration of the existence of alternation, as the response in the majority of fibres often remains in phase, thus reducing to one-half the frequency of response of the whole nerve (figs. 11, 12, Plate 2). This we consider to constitute strong evidence in favour of the alternation explanation of the reproduction of high stimulus frequencies in the mammalian case. In the insect also, the reproduction, even momentary, of a frequency as high as 6-800/sec imposes the assumption that the true phenomenon of alternation is sometimes being manifested. That is to say, owing to variations in the relative refractoriness of different fibres, the impulses in certain individual fibres (after the initial "on-effect") occur in response to alternate waves of the stimulus (*cf.* Davis, 1925, p. 209). Evidence for this supposition is provided by the curve connecting maximum response amplitude and stimulus frequency, which exhibits a drop to perhaps one-half of its original height at *circa* 200 c/sec.* Owing to the phenomenon of frequency-halving, we find no further steps of this type in the curve at higher frequencies, for, though the mean response frequency of the individual fibres is reduced to a submultiple of the stimulus frequency, the fact that the majority of the fibres still respond simultaneously may, when this occurs, lead to a rise in response amplitude (*cf.* figs. 11, 12, Plate 2). Thus we obtain a halving of response frequency at several

* This figure varies somewhat in different preparations, being dependent no doubt upon temperature, age of preparation, etc. In all cases, however, the drop occurs between 150 and 300 c/sec.

points in such a curve. The parallel between the response of the cercal nerve and that of the comparable mammalian preparation is even more obvious when it is realized that each exhibits *equilibration** of both the fast and slow types (see fig. 6, Plate 1, and cf. Davis, 1935, fig. 6). The latter we have investigated in the following way. The response to a note of constant intensity was photographed on continuously moving sensitive material. The stimulus was continued until the amplitude of the response showed no further change. The ensuing process of recovery was recorded by the momentary application of test stimuli of the same

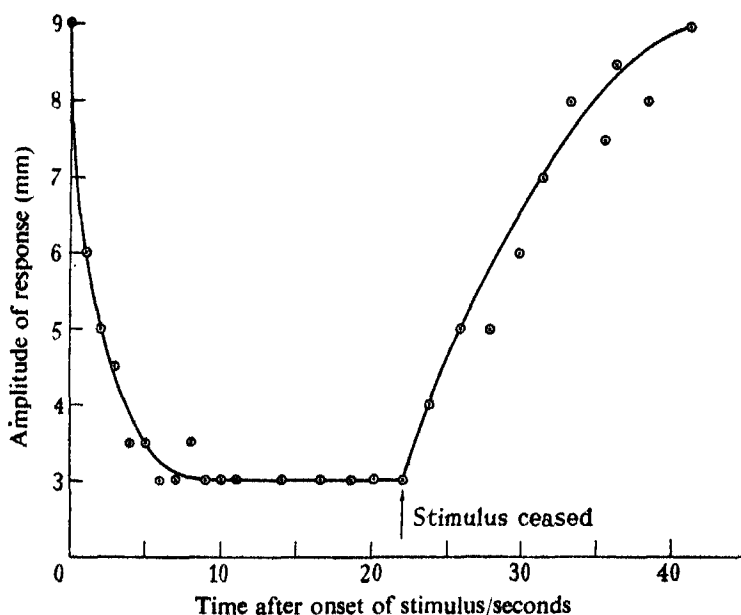


FIG. 7.—Graph to demonstrate equilibration in cercal nerve of *cockroach*. The intensity of the stimulus was constant throughout the experiment. Stimulus frequency, 300 c/sec.

frequency and intensity, the responses being again recorded photographically. This technique, precisely similar to that of Derbyshire and Davis, has yielded results also similar to theirs (see fig. 7, and cf. Derbyshire and Davis, 1935, fig. 4). As they do, we interpret these changes as indicating that, as the relative refractory period of each fibre lengthens (owing to repeated stimulation), so the number of impulses per unit wave of the stimulus decreases; moreover, the synchrony in individual fibres is partially lost as a result of sustained high-frequency stimulation of this type.

* The decline in amplitude of the response during a stimulus of constant intensity.

THE TYMPANAL ORGAN

For our investigation of the function of this organ we have used a locust.* In this animal there is a pair of organs situated at the sides of the first abdominal segment. Their histology and anatomical relations have been most fully described by Schwabe (1906). A condensed résumé may be given here. The tympanic membrane in a thin oval disk, about 1.0×1.5 mm, continuous at its edges with the exo-skeleton and protected on its external aspect by folds of the latter which form a primitive auditory meatus. The disk varies in thickness from 11μ anteriorly to 0.5μ near the posterior margin. It is underlain by the hypodermis, one cell thick, and in intimate contact with the inner surface of the latter is the external membrane of the outermost of three air sacs which together form a protective cushion for the auditory apparatus. In the disk there are three thickened regions, closely grouped and slightly anterior to the centre, which serve as points of attachment for three groups of chordotonal sensilla. These three groups contain about 8, 20, and 25 sensilla respectively. The afferent fibres from these join with the fibres from a few hair and possibly campaniform sensilla situated on one of the thickened regions of the disk; together these fibres form the tympanal nerve. This at first runs ventrally, enclosed in a fold of the external air sac, thereafter anteriorly to the third thoracic ganglion. On its course it is joined by a branch from the stigma immediately anterior to the tympanum and by a branch from the heart.

The nerve was exposed in the following manner. In an animal from which the head, wings, and legs had been removed, a flap, which included the tympanal organ of the left side, was removed from the body wall, making a window through which part of the gut was extracted. The last thoracic ganglion, together with the central end of the tympanal nerve of the right side, was then visible. This nerve was cut close to the ganglion and freed from connective tissue for a length sufficient to enable it to be placed on two fine electrodes.

Proceeding thus we elicited a response for any pure tone stimulus of sufficient intensity between 500 and 11,000 c/sec (fig. 8, Plate 2). At frequencies around 3000 cycles the organ appears somewhat more sensitive than elsewhere. The threshold at this frequency was found to be only about 20 db above the human threshold, corresponding to a power of

* *Locusta migratoria subsp. migratorioides*, in the gregarious phase. We are indebted to Dr. A. D. Imms for his courtesy in allowing us to use animals from cultures kept at the Entomological Field Station. The crickets used in the experiments described above are from the same source.

about 10^{-7} microwatts per square cm. So high a sensitivity for so simple an auditory organ is worthy of note.

At all frequencies and intensities which we have employed (up to 100 microwatts/sq cm) we have been unable to detect any trace of synchronization of the response to the stimulus frequency. This finding may be contrasted with that of Davis and his co-workers on the mammalian cochlear nerve, where the response is synchronous, provided at least that the stimulus frequency is below 3000–4000 c/sec, and with our own findings on the cercal nerve. It is worthy of note that the nerve from the tympanum of the locust contains only some 50–60 fibres. It is not impossible that the absence of synchrony, and the resultant absence of equilibration as the synchrony breaks down, may be ascribed to this fact. On this point, we find, indeed, that the initial amplitude of the response for constant stimulus intensity is maintained for 30 seconds or more, in striking contrast to the response of the cercal and cochlear nerves.

We are inclined on the basis of these experiments to deny to the locust the ability to discriminate frequency by means of its tympanic organ. There is no differentiation of nervous response for different frequencies of stimulus; and, moreover, it seems unlikely that the physical structure of this organ is such as would permit the discrimination of frequency by means of a differentially resonant mechanism. Since the organs are paired and, for sound at low absolute intensities, very sensitive to intensity fluctuations, they are well suited to enabling the insect to localize the source of a sound whose frequency falls within its auditory spectrum, *e.g.*, the stridulation of another locust.

On the other hand, it seems possible that the acoustic function of the cerci of the cricket and cockroach is merely incidental. Whilst the difficulty of distinguishing between acoustic and vibratory stimuli for an organ such as the cercus is fully realized, the relative insensitivity of the organ to all but the lowest frequencies, together with the fact that in the cricket it is frequently in contact with ground when the animal is in its normal habitat, leads us to suppose that its main function here is to mediate the detection of earth-borne vibrations. In the cockroach, where the cercus is carried more or less erect, it seems likely that its function as a wind-gauge may be equal in importance to its function as an acoustic organ.

SUMMARY

Evidence is produced to show that the anal cercus of the cricket and cockroach has a partly auditory function. This organ is physiologically of great interest, for there is a close parallel between the response in the

cercal nerve and that in the mammalian VIIIth nerve. The reproduction in the nervous response of relatively high stimulus frequencies, together with the phenomena of "frequency-halving", indicates the existence of "alternation", whilst "equilibration" of both the rapid and slow types may be demonstrated at the higher stimulus frequencies. These effects provide corroborative evidence of the alternation hypothesis in the mammalian case.

The nervous response of the tympanic organ of the locust is, on the other hand, asynchronous at all stimulus frequencies, and this organ resembles the mammalian ear only in its relatively greater sensitivity to the higher frequencies of the auditory spectrum.

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DESCRIPTION OF PLATES

PLATE 1

- FIG. 1—Response from cercal nerve of *cricket* to light puff of air. Time, 20 milliseconds.
 FIG. 3—Synchronous response from cercal nerve of *cricket* to pure tone stimuli of frequency (a) 100, and (b) 300 c/sec. Time, 20 milliseconds.
 FIG. 4—Response from the cercal nerve of *cricket* to pure tone stimulus (900 c/sec). Note absence of synchrony. Time, 20 milliseconds.
 FIG. 5—Initial response of cercal nerve of *cricket* to 400 c/sec pure tone. Note that response consists of volleys at 800 p.s. Time, 10 milliseconds.
 FIG. 6—Response from cercal nerve of *cockroach* to pure tone stimulus of frequency 300 c/sec and constant intensity. Note decline in response during first half-second to an almost constant amplitude (rapid equilibration). Time, 200 milliseconds.

PLATE 2

- FIG. 8—Response from tympanic nerve of *locust* to a pure tone stimulus of frequency 3000 c/sec. Time, 20 milliseconds.



FIG. 1.



FIG. 3.

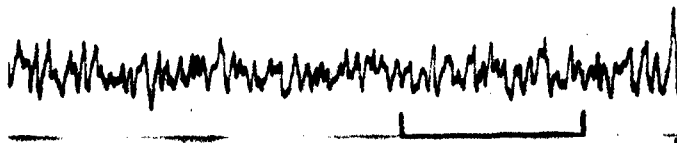


FIG. 4.

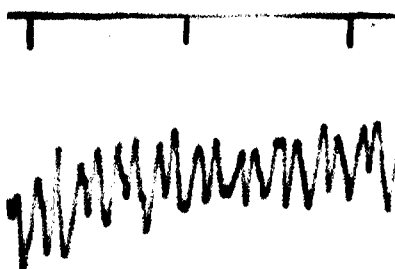


FIG. 5.



FIG. 6.



FIG. 8.

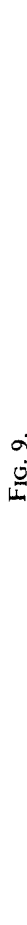
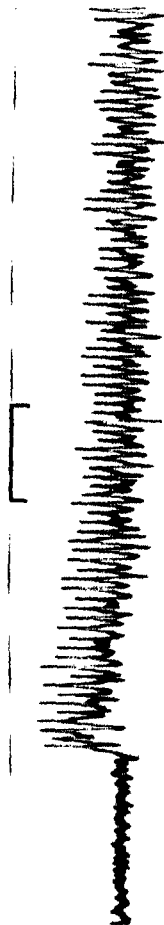


FIG. 9.

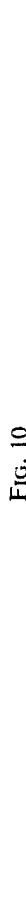


FIG. 10.



FIG. 11.

FIG. 12.

FIG. 9—Synchronous response from cercal nerve of *cricket* to a note of 500 c/sec immediately after onset of stimulus.

FIG. 10—End of same record as fig. 9. 0.33 sec elapses between the end of 9 and the beginning of 10. Note that in 10 the synchrony of response to stimulus has largely broken down.

FIGS 11 and 12—Section of continuous record of response of cercal nerve of *cricket* to short duration sound stimuli.

In fig. 11 the response to a note of frequency 600 c/sec is partly synchronous.

In fig. 12 the response to a 610 c/sec tone exhibits *frequency-halving*. Note that, disregarding the "on-effect", the amplitude of fig. 12 is greater than that of fig. 11. Response maximal in both cases.

In figs. 9 to 12 the time interval marked is equal to 20 milliseconds.

581.114.087

The Diffusion Porometer

By F. G. GREGORY and J. I. ARMSTRONG

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Various devices have from time to time been described for investigating the movement of stomata. These depend for their action on the rate of flow of air under uniform pressure drawn or pushed through the stomata; the measure of stomatal aperture is thus some function of the rate of flow. This principle was first used by Dutrochet (1832), and by Müller, in 1872, and an apparatus, using a cup attached to a leaf, was constructed by Darwin and Pertz in 1912. Self-recording porometers on the principle of Darwin's porometer were perfected by Balls (1912), Knight (1915, 1922), Laidlaw and Knight (1916), and Neilson-Jones (1914). For use with amphistomatous leaves a double porometer has been described by Leick (1928).

All the instruments mentioned measure the rate of flow of the gas. Recently a porometer has been described by Gregory and Pearce (1934) in which the fall in pressure of the air on passing through the leaf is the basis of measurement and nearly instantaneous readings of stomatal aperture are thus possible.

The conversion of these measures of stomatal aperture into terms of

change of diffusive capacity presents a problem which has not yet been completely resolved by experimental methods. It would therefore appear that a method of direct estimation of the diffusive capacity of the stomata is very desirable.

The present paper describes an apparatus which makes possible a direct and continuous measure of the change in the diffusive capacity of stomata during their opening and closing.

PRINCIPLE OF THE METHOD

In principle the method employed depends on the fact that the rate of diffusion of gases is inversely proportional to the square root of their densities. If therefore a porometer cup, attached to a leaf in the usual way, is filled with hydrogen at atmospheric pressure, and connected directly to a manometer, then the rate of diffusion of hydrogen outwards is greater than the diffusion of air inwards and an increasing negative pressure is developed. The application of the principle was first tested in this way and it was found that in practice the method afforded the basis for a diffusion porometer.

The original apparatus is shown diagrammatically in fig. 1. The porometer cup P was attached to the leaf by means of a gelatine washer W, and the whole apparatus was filled with hydrogen by opening the stop-cock A and turning B into connexion with an escape trap E and manometer M. To take a measurement the tap A is closed from communication with the supply of hydrogen and the tap B is turned to bring the manometer only into connexion with the porometer cup. The diffusion of the hydrogen leads to the development of a negative pressure and the liquid in the manometer rises, rapidly at first then more slowly, later a maximum is reached after which the level of the liquid gradually falls. The result of such an experiment is shown graphically in fig. 2 and the values entered in Table I.

Many experiments were carried out with this apparatus with various leaves, and with stomata at different stages of opening. It was found that the maximum reading of the manometer was related to the degree of opening of the stomata; lower maxima were obtained with wide open stomata, the rate of rise in these cases being greater. The effect of the stomatal opening was very marked; with the stomata apparently completely closed the time required to reach the maximum pressure was a matter of some hours, while, with the stomata fully opened the time required was of the order of 10 minutes.

The form of the curve shown in fig. 2 is determined by the following factors:—

(1) The rapid fall in pressure is determined by the rate of diffusion of the hydrogen out of the system.

(2) As diffusion proceeds there is a fall in the concentration of hydrogen tending to reduce the outward diffusion.

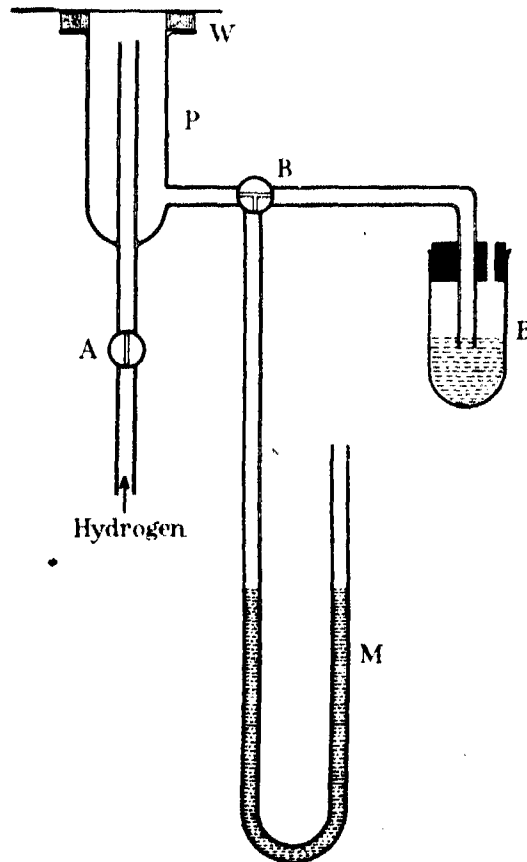


FIG. 1—Apparatus for demonstrating hydrogen diffusion by pressure change.

(3) A back diffusion of air occurs, most rapid at first and tending to fall.

(4) As the negative pressure develops, a mass flow of air into the system occurs determined by the negative pressure within as in an ordinary flow porometer. The tendency for pressure to fall due to (1) is thus opposed by the processes (3) and (4), and results in a maximum point of negative pressure at which outward diffusion of hydrogen is just balanced.

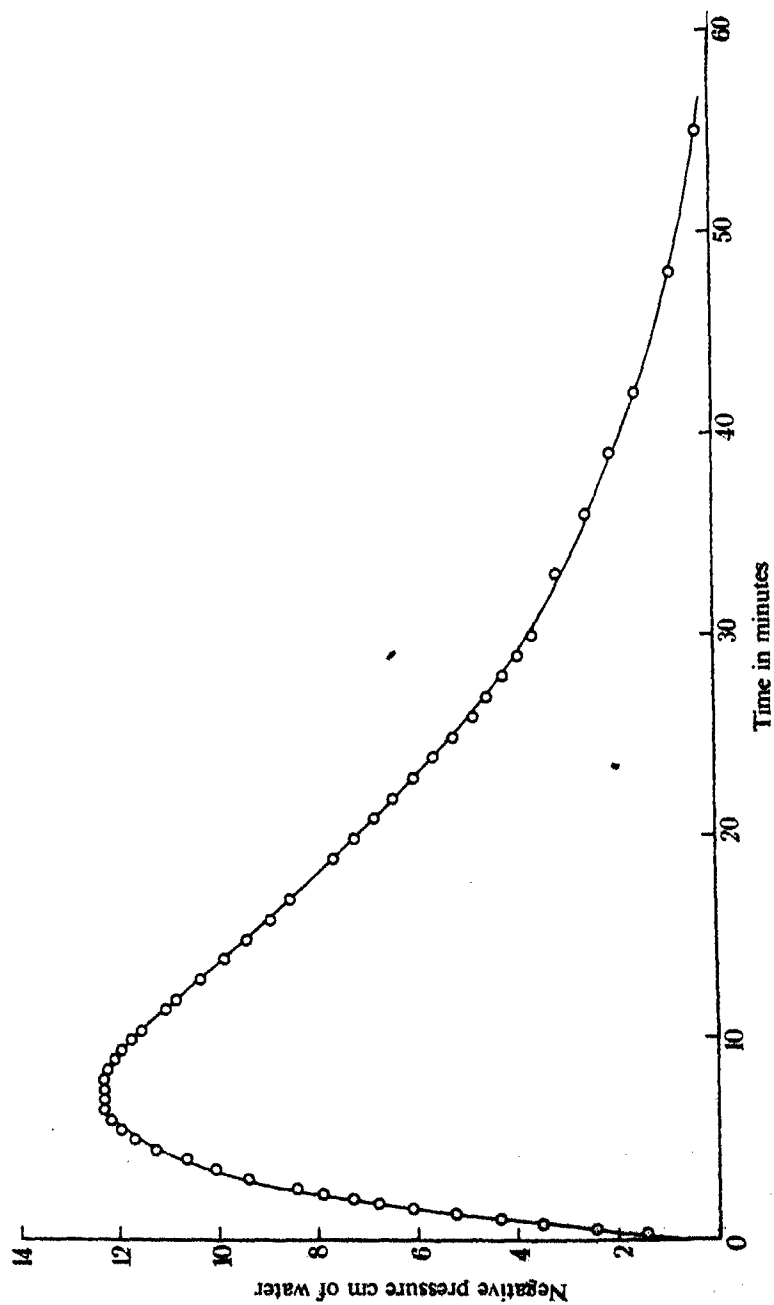


FIG. 2.—Record of pressure changes during progress of hydrogen diffusion through a leaf of *Pelargonium zonale*.

by the inflow of air. The maximum point is determined partly by the decrease in the concentration of hydrogen and partly by mass flow of air under the negative pressure developed. That pressure changes in the system were due to diffusion through the leaf, and not through the rubber connexions nor at the gelatine washer, was shown by attaching the porometer cup to a glass plate. In 47 minutes a negative pressure of only 9.5 mm developed.

TABLE I—HYDROGEN DIFFUSION WITH A LEAF OF *Pelargonium zonale*

Time in min.	Negative pressure (cm water)	Time in min.	Negative pressure (cm water)
$\frac{1}{2}$	1.4	11 $\frac{1}{2}$	11.0
$\frac{1}{2}$	2.5	12	10.8
$\frac{3}{4}$	3.5	13	10.3
1	4.4	14	9.8
1 $\frac{1}{4}$	5.3	15	9.4
1 $\frac{1}{2}$	6.1	16	8.9
1 $\frac{3}{4}$	6.8	17	8.5
2	7.4	—	—
2 $\frac{1}{4}$	8.0	19	7.6
2 $\frac{1}{2}$	8.5	20	7.2
3	9.5	21	6.8
3 $\frac{1}{2}$	10.2	22	6.4
4	10.8	23	6.0
4 $\frac{1}{2}$	11.3	24	5.6
5	11.7	25	5.2
5 $\frac{1}{2}$	12.0	26	4.8
6	12.2	27	4.5
6 $\frac{1}{2}$	12.3	28	4.2
7	12.3	29	3.9
7 $\frac{1}{2}$	12.3	30	3.6
8	12.3	33	3.1
8 $\frac{1}{2}$	12.2	36	2.5
9	12.05	39	2.0
9 $\frac{1}{2}$	11.9	42	1.5
10	11.7	48	0.8
10 $\frac{1}{2}$	11.5	55	0.2

Although the apparatus already described was serviceable, it was soon realized that no simple measure of stomatal aperture could be obtained as the maximum pressure reached would depend on the area of the porometer cup and on its volume. Further, since the maximum depends also on mass flow, the same difficulties would be encountered as in the interpretation of records of the flow porometer. To overcome the difficulty, a null method was devised in which the hydrogen diffusing from the cup

is replaced by electrolytic hydrogen so that no change of pressure develops in the porometer cup; the rate of diffusion now equals the rate of supply of hydrogen. Thus by simply measuring the current used in electrolysis the rate of diffusion can be determined.

DESCRIPTION OF THE DIFFUSION POROMETER

The construction of the apparatus is seen from a diagrammatic representation in fig. 3. The porometer cup P is attached to the leaf by means of glue or a gelatine washer, and from a large electrolytic generator the main hydrogen supply is led to the porometer cup through the tap A. The small secondary supply enters through the three-way tap C which also communicates with an escape trap E; this supply is provided by the subsidiary generator which consists of two small electrodes F and G immersed in a saturated solution of barium hydroxide. The anode F, from which oxygen is liberated, consists of a platinum plate sealing the end of a glass tube containing mercury, for electrical contact. The oxygen escapes to the air from the immersion vessel through a soda lime tube. The cathode G consists of a long spiral of platinum wire contained in a glass tube closed at the end by a sintered glass plate. This plate is necessary to prevent the baryta being driven from the electrode tube by the hydrogen generated during preliminary adjustments, but it does not prevent the flow of the current. The electrolysis apparatus is surrounded by a water bath to minimize temperature changes. The manometer M is connected by the two-way tap B to the porometer, and also, when necessary, to the air for the purpose of zero adjustment. The quantity of hydrogen generated by the secondary electrode is regulated by a sliding resistance and a resistance box, the current being recorded by a milliammeter.

The apparatus is used in the following way. First, by opening tap A to the main generator and tap C to the escape trap, the apparatus is swept clear of air. The tap A is then closed, C opened to the secondary electrode and B to the manometer. If the secondary supply of hydrogen is not switched on, the liquid in the manometer rises, reaches a maximum, and falls, as before. When the loss of hydrogen is to be balanced the level of liquid in the manometer is observed through a telescope and the secondary generator switched on. If the current passing is too great the level in the manometer falls owing to the excess pressure developed, and if too small a current is used the liquid rises. By trial and error the sliding resistance is so adjusted that, after closing the main hydrogen supply,

and immediately switching on the secondary supply, no change in level of the manometer results. From the reading of the ammeter the rate of diffusion of the hydrogen is directly calculated.

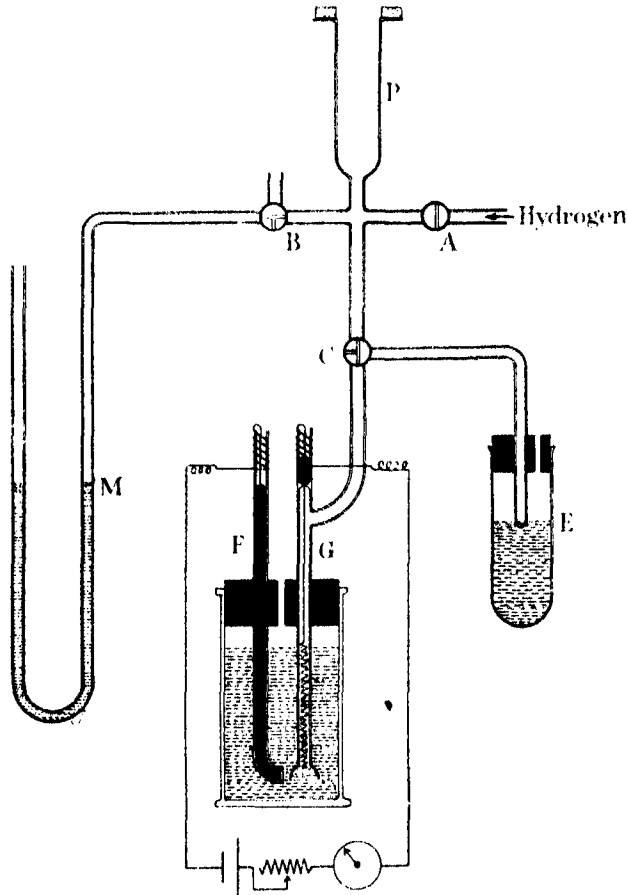


FIG. 3—Final form of diffusion porometer. Pressure change is maintained constant by replacing hydrogen diffusing out of porometer cup (P) by hydrogen from auxiliary electrode (G). Diffusion rate is measured in terms of the current flowing to auxiliary electrode.

PRELIMINARY TESTS OF THE METHOD

1—*Effect of Hydrogen on Stomatal Movement*—The effect of hydrogen in the intercellular spaces of the leaf was investigated, using *Pelargonium* leaves attached to the plant and enclosed in a glass chamber through which hydrogen could be passed. The porometer cup passed, with an airtight joint, through the base of the chamber and was attached to the leaf

with a gelatine washer. To secure adequate contact with the washer the leaf was pressed against the upper side of the glass chamber. Continuous records of stomatal aperture were made by means of an electrical recording porometer as described by Knight (1915), the whole porometer and aspirator being filled with hydrogen. Two such experiments were carried out; the records of gas flow (l/T ; T = time per bubble) of one such experiment are shown in fig. 4.

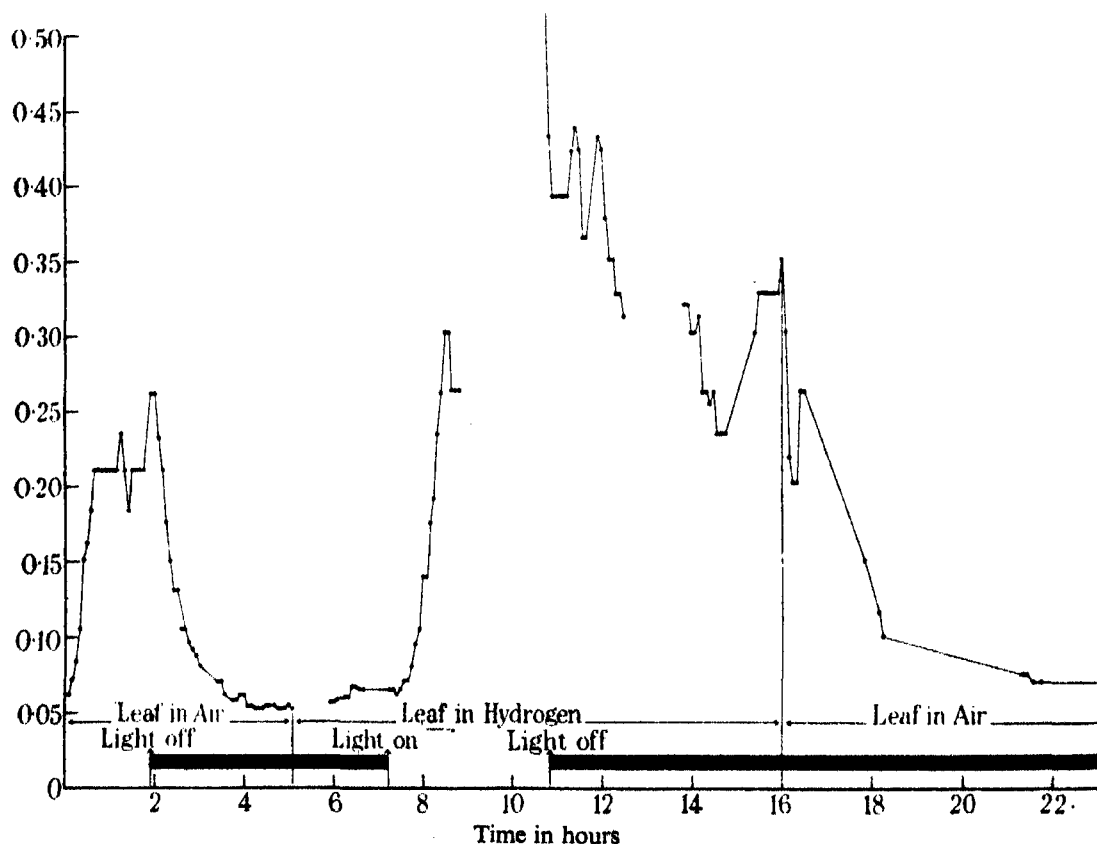


FIG. 4—Record of stomatal movement of leaf in hydrogen and in air.

In the first part of the experiment *air* was passed through the chamber. After being kept in the dark overnight when the light was switched on the stomata opened fully in 40 minutes and remained unchanged until the light was switched off after 2 hours. Stomatal closure then began and was complete after a further 2 hours. After 5 hours from the start the chamber and the porometer were filled with hydrogen and 1 hour later the light was again switched on, hydrogen passing continually through the

chamber. The stomata again opened fully in 60 minutes and attained a somewhat wider opening than previously in air. In 10½ hours from the start the light was switched off; the stomata, however, failed to close and remained wide open for a further 6 hours. The hydrogen in the chamber was now replaced by air, and complete closure of the stomata then occurred. Similar results were obtained with the diffusion porometer in its original form. As in the early experiments commercial hydrogen from a gas cylinder was used it was thought that possibly some toxic impurity in the hydrogen was responsible for the failure to close, so various washing devices were tried but without success. Hydrogen was then generated by electrolysing a saturated solution of barium hydroxide; but still even in pure hydrogen failure of the stomata to close in the dark was again observed. It was further noted that exposure of the leaves in the dark to short periods (5 minutes) of air was not sufficient to induce closure. In later experiments detached leaves were used and it was found that partial closure would take place in hydrogen in the dark. A further interesting fact was noted that in contrast with *Pelargonium*, *Fuchsia* leaves always showed stomatal closure in hydrogen whether the leaves were detached or not. These observations have not been further pursued, so the relations of stomatal closure to oxygen supply still remain to be determined. It would, however, appear that anaerobic conditions inhibit the closing mechanism of some leaves under certain circumstances.

TABLE II

Time (min)	mA
0	87.5
5	62.5
10	55
15	45
20	40
30	32
40	25

2—*Backflow of Air*—The initial backflow of air on shutting off the supply of hydrogen will tend to increase the pressure within the system. If, therefore, the loss of hydrogen has been balanced by extra hydrogen supply from the secondary electrode, and the current is kept flowing, the pressure in the manometer will continually rise, and to maintain the manometer at zero the current will continually need to be reduced. That this is so is shown from the values in Table II, where it is seen that the current necessary to balance the outflow of hydrogen falls more or less

logarithmically with time. These results were obtained with the use of a porous pot in place of the leaf, and similar results have been obtained with leaves. Since, in practice, the time occupied by a reading is at most 10 seconds the error introduced into the determination of the initial rate of hydrogen diffusion is very small. Where high accuracy is required, the "balancing" current values can be obtained by extrapolating back to zero by plotting the logarithms against time, and the initial current is read from the graph.

3—*Effect of Diffusion of Hydrogen Within the Cup*—The hydrogen diffusing through the lower epidermis of the leaf has to be replaced by diffusion within the whole system, so diffusion within the cup might limit the rate of diffusion outwards. In order to investigate this point, experimentally, the apparatus shown in fig. 5 was used.

A metal plate M was drilled with a circular hole equal in diameter to that of a narrow porometer cup P. The metal was attached to the leaf by means of the gelatine washer W_1 ; a second gelatine washer W_2 made contact between the metal plate and the porometer cup. A considerably wider cup C was arranged concentrically with P, and was permanently attached to the metal plate. The porometer cup P could be moved to make contact with the plate M, thus closing off the large cup, or could be withdrawn to bring the larger cup also into connexion with the leaf. The area through which diffusion took place thus remained constant, while the mass of hydrogen diffusing could thus be varied in the manner indicated. This double cup was tested on several leaves, and it was found that the rate of diffusion of the hydrogen outwards was independent of the volume of the cup used, and therefore of the relation between diffusing area and the mass of hydrogen.

4—*Effect of the Diffusion Gradient*—In order to test whether the rate of diffusion into the leaf was, in fact, proportional to the gradient of hydrogen, the following experiment was carried out. The leaf was enclosed in a chamber through which mixtures of hydrogen and air, of known hydrogen concentration, could be passed. The porometer cup passed into the chamber by an air-tight joint, and was attached to the leaf in the usual way. A leaf with wide-open stomata was used for the test and by the null method the rate of diffusion of hydrogen determined for different concentrations of hydrogen in the chamber. At intervals during the experiment the rate of diffusion in air was estimated to ensure that no stomatal movement had occurred. The results of the experiment are shown graphically in fig. 6, and it is seen that the rate of diffusion of the

hydrogen through the leaf is strictly proportional to the concentration gradient of hydrogen, proving that the diffusion rate is being studied.

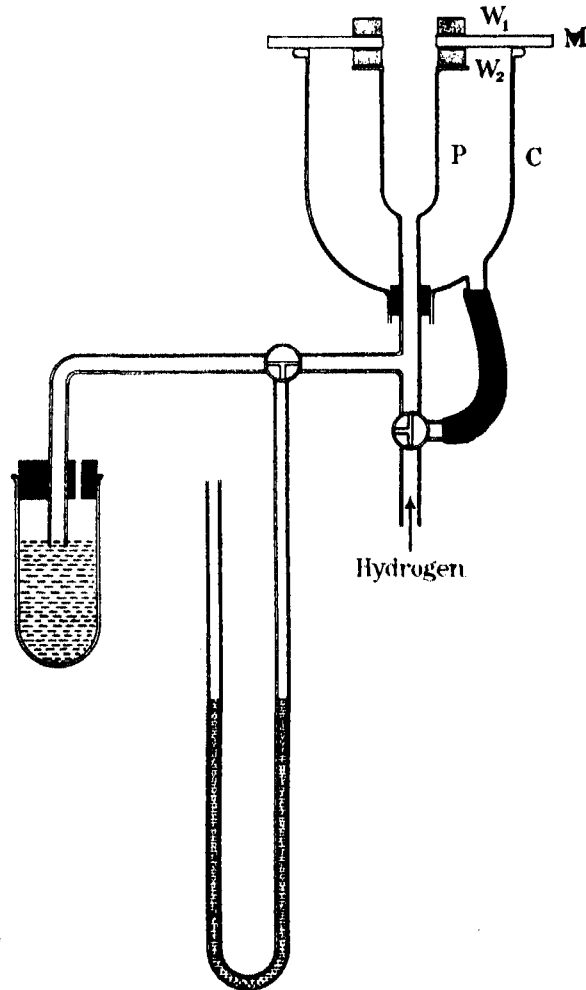


FIG. 5—Double porometer cup to investigate the effect of the volume of the cup on the diffusion rate of hydrogen through the leaf.

EFFECT OF STOMATAL APERTURE ON DIFFUSION RATE

In order to show the effect of stomatal aperture on the diffusion rate, the results of a typical experiment are presented in Table III and graphically in fig. 7. A leaf of *Fuchsia* detached from the plant was used, and the cup attached with gum at 1 p.m., $2\frac{1}{2}$ hours before the light was switched on.

The values given in Table III represent the current (milliamps) passing

through the balancing electrode. It will be noted that the first value given prior to switching on the light shows some diffusion of hydrogen, and it was always found that even when the stomata appear closed a slow diffusion of hydrogen occurred. How far this may be due to cuticular diffusion is not known.

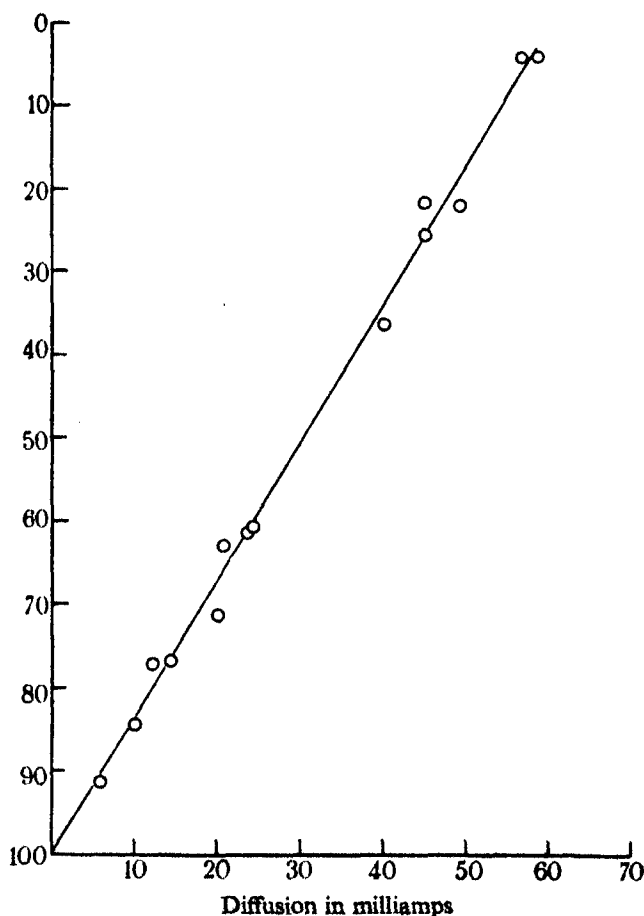


FIG. 6—Relation between the rate of diffusion through the leaf and the external partial pressure of hydrogen.

COMPARISON OF THE DIFFUSION POROMETER WITH THE RESISTANCE POROMETER

The relation between mass flow of air and the diffusion of hydrogen for a given stomatal aperture was tested by using, on the same leaf, the diffusion porometer here described, and the resistance porometer of

TABLE III

	Time min	mA
Light on	0	15
	5	40
	10	55
	15	90
	28	90
	39	90
	43	90
Light off	47	87.5
	54	75
	62	52.5
	67	45
	72	35
	81	25
	94	20
	115	20
	123	15

Leaf left open to the air overnight, and the light switched on at 10.23 a.m.

Light on	0	32.5
	11	50
	23	75
	32	87.5
	45	93
	54	95
Light off	67	90
	74	70
	81	50
	106	32.5
	146	25

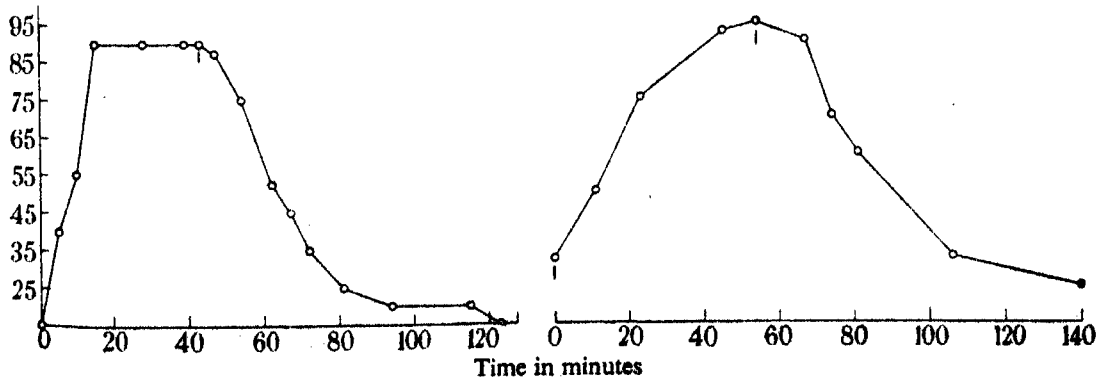


FIG. 7—Record of variation in hydrogen diffusion with stomatal aperture (*Fuchsia*).

Gregory and Pearse (1934). The porometer cup was arranged so that it could be put in connexion with either of the porometers, and thus both the diffusion rate and the resistance to mass flow could be determined. Direct comparison in this way, using the same cup, was possible over a wide range of stomatal aperture, but when the stomata were nearly closed the method failed since the time taken to reach equilibrium in the resistance porometer was now considerable, and therefore considerable changes in the diffusive capacity took place in the interval so that the results were not directly comparable. To investigate nearly-closed stomata two cups were used, side by side on the same leaf, and attached to the two porometers.

The results of these experiments are graphically presented in fig. 8. The results of several experiments are indicated by different signs in the figure. The curve approximates very closely to a hyperbola, and the curve drawn was calculated as the hyperbola of closest fit. It will be seen that for small openings of the stomata the flow relative to the diffusion increases very rapidly, while with wide open stomata the reverse is true. The relation between diffusion, flow resistance, and stomatal aperture will be considered in a subsequent paper.

The graph presented in fig. 8 may be used as a calibration curve which enables stomatal aperture, measured in terms of resistance (capillary units), to be converted into diffusive capacity in terms of milliamps, or alternatively as grams of hydrogen diffusing through unit area of the leaf under a pressure of 1 atmosphere.

SUMMARY

The paper discusses a method for determining experimentally the relative diffusive capacity of stomata at different apertures. The method depends upon the difference in rate of diffusion through the stomata of hydrogen and air.

An instrument is described embodying this principle. A porometer cup is attached to the leaf and connected with a supply of hydrogen and with a manometer. On cutting off the supply of hydrogen the diffusion of the hydrogen through the stomata causes pressure changes in the system which are recorded.

Experimental diffusion curves are presented and the pressure changes found are discussed in relation to diffusion of hydrogen outwards, back diffusion of air, and mass flow of air due to the negative pressure developed.

To overcome the effect of back diffusion of air and mass flow under the negative pressure developed, a null method was devised. The outward

diffusion of hydrogen is balanced by adding hydrogen to the system, the hydrogen being produced by electrolysis. A constant pressure equal to that of the atmosphere is thus maintained within the porometer cup.

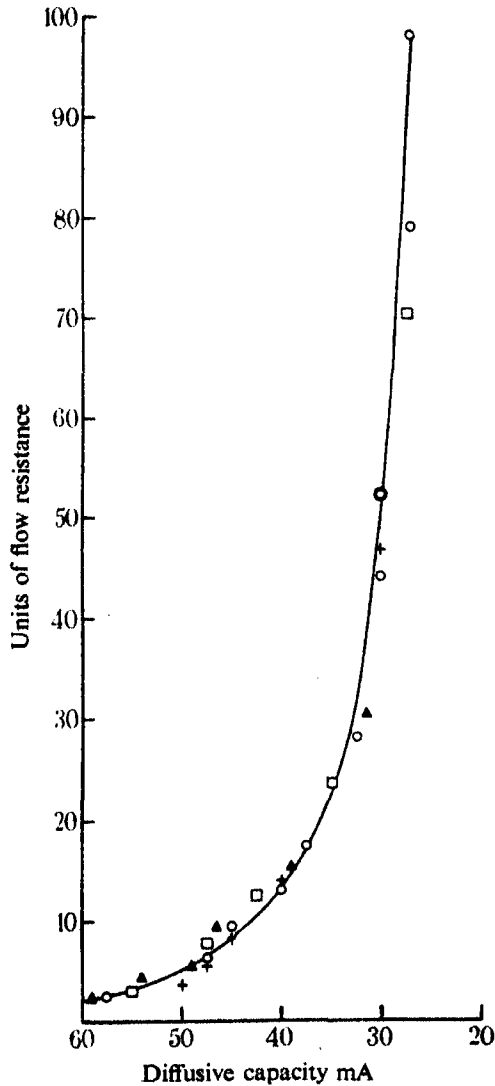


FIG. 8—Relation of flow resistance to diffusive capacity at varying stomatal aperture (*Pelargonium*). Four experimental records are included.

The final form of the porometer based on this null method is described. The diffusive capacity of the stomata is measured by determining the current required to produce the hydrogen necessary to maintain the pressure in the system.

The sources of error of the instrument are discussed and proof is presented that the diffusion rate of hydrogen through the stomata is actually measured.

Typical curves of diffusive capacity for opening and closing of stomata are presented. A failure of stomata to close in hydrogen under some conditions is noted.

A comparison between the diffusion capacity of stomata at varying aperture and the flow resistance as determined by the resistance porometer (Gregory and Pearse) is shown graphically.

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A Discussion on the Present State of the Theory of Natural Selection

OPENING ADDRESS

By Professor D. M. S. WATSON, F.R.S.

“ Natural selection or the preservation of favoured races in the struggle for life ” is an explanation primarily of adaptation and only secondarily of the origin of species.

It rests on the assumption that the differences between individual members of a species which live together in the same district are heritable, and that they may determine the life or death of the individuals in question at an earlier or later time than one another. In other words, that definite variation in structure or in function may be responsible for a selective death-rate.

It is evident that this selective death-rate will only be recognizable statistically, and that a heavy non-selective infantile death-rate, such as that which occurs generally in marine teleosts, merely reduces the population within which selection of adults may operate.

The existence of such a selective mortality is fundamental to the theory, and various authors, from the time of Weldon, have tried to establish its occurrence in definite cases, always without any significant result.

It is presumably true that relatively gross abnormalities lead to early death, but in some cases (*e.g.*, *Rhynchonella*) the average size of asymmetrical shells seems, on a mere inspection, to be no smaller than that of normal individuals from the same horizon and locality.

It would thus be valuable to have any new evidence on this point, or indeed any suggestion of cases which might be capable of investigation.

The nature of the variations which do occur in nature and which may provide the raw material of evolution, requires experimental investigation.

The varieties recognized by systematists, are, I think rightly, held by them to include two types: those which occur within a single population, and which therefore cannot be accounted for by environmental effects (except for those special cases involving microclimates), and those which are assumed to be related to local variations in the environment, and are hence of a geographical significance.

In some special cases, *e.g.*, the spring and summer broods of moths, the varieties are clearly the result of climatic changes, but in most, *e.g.*,

the commonly darker colour of forest mammals in contrast with those from open country, either a direct environmental influence or a Darwinian explanation are possible, and no adequate experiments designed to choose between them have yet been carried out.

The extent to which these naturally occurring variations are heritable, and the nature of their heredity have seldom been investigated. We do not know how often mutants occur in nature either as phenotypes or in the form of recessive genes hidden by their existence in heterozygotes.

If certain assumptions be made it is possible to calculate the number of individuals possessing a recessive gene in the homozygous condition which would be expected to occur in a population containing a known proportion of heterozygote individuals carrying that gene, and a comparison between that calculated number and those actually exhibiting the character which can be caught wild might be an effective method of determining the relative viability of the two types under natural conditions, although any differences discovered will not necessarily be dependent on the visible differences, and hence will not provide a measure of the natural selection dependent on such observed characters.

One of the most striking facts which has emerged from palaeontological studies is that the same structure may arise, apparently for an adaptive end, repeatedly and independently in animals which are nearly allied, whilst the same ends may be served by quite different adaptations in other groups. A familiar example is the molarization of premolars in Perissodactyls to secure an increased grinding surface, whilst the Artiodactyls living on the same foodstuffs in the same areas and at the same times never exhibit this phenomenon.

It is important to discover whether the same set of genes are responsible for the occurrence of such identical structural changes throughout a group, or whether their homology does not extend to that of the hereditary mechanism which determines them.

In many cases great structural changes which have every appearance of being adaptive, the changes in the dentition of the horses from *Hyracotherium* to *Equus*, for example, seem to be related to increase in size; they enable a large animal to live as long as a small one on food of the same nature, and the governing consideration would be the greater overall efficiency which a large mammal can secure.

Such cases raise in an acute form the problem of the coordination of the evolutionary changes which is necessary to ensure that animals remain workable wholes.

It is probable that the differences between geographical races (which have only a statistical meaning) have no adaptive significance, and it is

important to know whether such races may be regarded as representing a steady state resulting from the isolation of a small initial group of individuals containing only a selection of the genes present in the population from which they were derived.

Finally it is clearly desirable that attempts should be made to discover the nature of the differences between the gene content of members of closely related species.

The theory of natural selection is the only explanation of the production of adaptations which is consonant with modern work on heredity, but it suffers from the drawback that by the introduction of related subsidiary hypotheses it becomes capable of giving a theoretical explanation of any conceivable occurrence, and that the scope and indeed the validity of its basal assumption of a selective death-rate determined by a favourable variation have not yet been established.

Professor N. W. TIMOFEEFF-RESSOVSKY (Berlin-Buch)—*Some Genetic Experiments on Relative Viability*—Experiments designed to determine the relative rates of hatching of a certain inbred culture of *Drosophila melanogaster* of populations of *D. funebris* from different areas were carried out by starting culture vials with equal numbers of the eggs of the two species the total number of eggs constituting severe overcrowding. The results were as follows.

In general the relative viability (expressed in rates of hatching) of *Drosophila melanogaster* is higher than that of *Drosophila funebris*. But at a low temperature (15° C) the hatching-rate of *D. funebris* reaches and even exceeds that of *D. melanogaster*. This is in good conformity with the geographical distribution and ecology of these two species, of which *D. funebris* is adapted to a cooler climate. *D. funebris* from geographically different populations (from different parts of Europe, from northern Africa, Caucasus, Turkestan, and western Siberia) showed rather small differences in relative viability at normal temperature (22° C), but could be grouped into three distinct "temperature races" according to their relative viability in low (15° C) and high (29° C) temperatures: the populations from north-western Europe showed a lower relative viability at high temperature, those from the Mediterranean districts showed lower relative viability at low temperature, and the populations from eastern Europe, Caucasus, Turkestan, and Siberia showed higher relative viability both at low and at high temperatures. This corresponds very well with the climatic characteristics of the region from which they were derived, and is thus a case of a physiological adaptation to the environment which is clearly heritable.

These adaptations can be explained in terms of Natural Selection, if it be admitted that they are due to the selection of small physiological mutations, affecting the relative viability of the flies. Such a selectionistic explanation is, however, based on three main premisses: (1) that mutations are present (in heterozygous condition) in free-living, wild populations; (2) that gene-mutations really do affect the relative viability of the organism; and (3) that "small physiological" mutations really do occur in *Drosophila*. These premisses can be tested experimentally.

In inbreeding fertilized wild females of *Drosophila melanogaster* from a free-living population (near Berlin) we found in 1927 that this population contained at least 12 different mutations (some of them in rather high concentrations) in heterozygous condition. Approximately at the same time similar results were obtained by Tshetverikov and his assistants, in analysing in the same way a population of *Drosophila melanogaster* from the Caucasus. Afterwards substantially the same results were obtained by Dubinin and his co-workers in different populations of *D. melanogaster*, by Gershenson in *D. obscura*, by Romashov and Balkashina in *D. phalerata*, *D. transversa*, and *D. vibrissina*, by Gordon in *D. melanogaster* and *D. subobscura*, by Romashov, and by myself in *D. funebris*. Thus, gene-mutations are abundantly present in heterozygous condition in every free-living population.

Experiments on the relative viability of different mutations and their combinations were performed on six sex-linked mutants in *Drosophila funebris*. The method consisted in the detection of deviations from the 1:1 ratio in backcrosses bred under definite constant conditions of overcrowding, food, and temperature. The results showed that different gross mutations exert specific influences on the relative viability of the flies in different environments, and in different combinations with other gross mutations and modifying factors. Most of them lower the relative viability of the wild type, but some of them can improve it under certain conditions, by displacing the temperature-optimum, or by improving the resistance to extremely overcrowded conditions. Thus, physiological adaptations can really be caused by gene-mutations.

The third premiss is whether "small physiological" mutations affecting the viability, and only slightly, if at all, affecting the morphological characters of the organism, do really occur in *Drosophila*. It was tested with the help of the X-ray method of accelerating the rate of mutation. Males of an inbred stock of *D. melanogaster* with a known sex-ratio (of about 1.00 ♀ : 0.95 ♂) were X-rayed and crossed to "C1B"-females from the same stock. Mutations affecting the relative viability, which may have arisen in the X-chromosome of treated males, will alter the sex-

ratio in corresponding F_2 -cultures. Their inheritance and sex-linkage can be proved by further crossings. The results of these experiments showed that mutations slightly affecting (in most cases lowering) the viability, arise at least twice as frequently as all other types of mutations (including the lethals) taken together, thus proving that the "small physiological" mutations are the commonest type of hereditary changes.

I think that the three main premisses of a selectionistic explanation of racial adaptations to the environment, have thus been proved.

Professor E. J. SALISBURY, F.R.S.—*Natural Selection and Competition*—Natural selection is one aspect, though only one, of the effects of competition amongst plants and animals. That competition is severe in the plant world no one can deny and this is operative at all phases of development. Whilst a very large proportion of the disseminules of species may never germinate, the proportion that are potentially viable is usually high, and the habitat conditions necessary for germination may and probably do exercise a certain selection unless the disseminules are strictly uniform in character, which in nature is probably unlikely. In some plants the archisporial tissue is multicellular and in others more than one potential embryo may occur in the embryo sac more commonly, but a proportion of the fertilized eggs develop embryos. All of which facts render probable the occurrence of some measure of selection prior to actual germination. Whilst, however, such pre-natal selection might be of considerable importance, the degree of its significance is as yet problematical.

The enormous number of potential progeny of some plants emphasizes the probability that they will not be genetically uniform, but that mutations may occur among them. The spore output of a single generation of *Lycoperdon bovista* has been estimated as of the order of 10^{12} , and even among flowering plants a seed production of 10^6 is by no means rare. The enormous mortality necessary to maintain the comparative secular stability of plant populations might in itself involve little if any selection. The ones which die need not necessarily differ genetically from those which survive. We know that almost any wild population of a species comprises strains and ecotypes, more rarely mutations, which under cultural conditions it is possible to segregate. It is reasonable to suppose that such differences do in fact affect the chances of survival, and indeed the common belief that cultivated species are more variable than wild ones, though partly due to hybridization, is largely a consequence of the fact that, under the diminished competition which obtains in artificial culture, together with the more favourable conditions of growth, many types survive that under the severity of natural conditions would inevitably

perish. In this connexion one may emphasize that in the British flora some of our most polymorphic species are common weeds of arable land where the artificial reduction of the severity of competition increases the possibility of survival of others than the most robust types. One need only cite the large number of hereditary types belonging to the aggregate *Senecio vulgaris*, a number of which were studied by Professor Trow, the numerous microspecies of *Capsella*, of *Viola arvensis*, or of *Taraxacum*. The precocious self-fertilization of *Capsella*, and the apomictic reproduction of *Taraxacum* exclude hybridization as an explanation of their multiplicity of types. An extensive experience with growing wild British plants from seed forces one to the belief that the surest way of obtaining uniformity is by growing dense cultures, whilst to ensure the survival of diverse types one must plant the seeds separately or plant out the seedlings at a very early stage.

If such microspecies be grown in mixed culture they show, as Sukatschew demonstrated for *Taraxacum officinale*, very different competitive ability. Mixed cultures in my own experience, whether of microspecies or *Linneons*, may show diminution of one or the other or even complete elimination, but the selective action of habitat conditions is rendered lethal by competition. We are then forced, I think, to admit that competition not only involves high seedling mortality but is often also selective.

Since the time of Darwin the principle has been enunciated that competition is most severe between individuals of the same species. This generalization is, however, misleading. I am aware of the difficulty of assessing severity of competition of which this generalization takes no cognizance. If we use the incidence of mortality as a crude measure of severity we must recognize that the densest populations in natural conditions are most frequently associated with almost pure stands of a single species. The explanation is, I suggest, to be found in the fact that in such communities the individuals are so evenly equipped that there is little if any actual elimination, though all the individuals may be depauperate. Competition between different varieties or species which are markedly different in their competitive equipment but at the same time make similar demands upon the habitat, is likely to be most severe and lead to the most pronounced selection. It is for this reason, probably, that in my own experience complete elimination is more frequent in mixed cultures of species than in cultures of different strains.

Observations, chiefly on annual species under both natural and artificial conditions, demonstrate that the seedling mortality is far in excess of that at any other period of the life history. Nevertheless, if this mortality were non-selective in character and the survivors, though few, formed a

representative sample of the population of strains originally present, it follows from the high reproductive capacity of plants that the juvenile mortality might not effect any change in the character of the population.

Cultures of annual species show that those individuals that survive the seedling stage mostly attain the adult condition. The mortality of the arboreal perennial is very high in the juvenile stage, but there is also a considerable mortality from suppression later in life, due mainly to what may be summed up as space competition. There is no evidence that this adult mortality is selective, the mortality appearing to depend upon the extrinsic variations in the habitat rather than on intrinsic qualities of the individuals. Selection in the adult stage, when it occurs, probably depends on factors affecting pollination, fertilization, and dispersal; in other terms, on those which affect the succeeding generation.

On general grounds it is reasonable to suppose that in a non-uniform population of seedlings the different types might be affected differently by adverse conditions. If the hereditary differences are of a qualitative character total elimination might ensue; if, however, of a quantitative character, as for instance in respect to stature or root development, then if the range of variation of the two types overlapped diminution rather than elimination of the one might be expected. It is, however, very important to emphasize in this connexion that since plant growth obeys the compound interest law a small initial difference between strains or species which gives advantage to either may soon become amplified in its effects to the point of suppression.

If we once admit the fundamental premiss that the greater part of selection as between plants of similar life form takes place in the seedling phase a most important corollary appears to follow, namely, that many of the adult characters of organisms may be only important as symptoms of the characters of survival value in the juvenile state with which they are associated. Perhaps only rarely, as in development of the root or the leaf-canopy, does the physiological equipment, that conditions survival, find direct expression in external morphology.

Dr. W. B. TURRILL—*Natural Selection and the Distribution of Plants*—The study of Natural Selection as one cause of the known distribution of existing plants involves consideration of two groups of variables: those of changing environments and those of varying, and to a certain extent evolving, plants and plant populations. It is important in distributional research to remember that no factor of the environment is invariable, or acts independently, that no individual plant is static, and that the content

of our more or less natural groupings of plants into taxonomic units, such as species, is continuously changing.

We know relatively little for the vast majority of plant species, of the habitat limits, of the variation of the species, or of the plasticity of the individuals under different environmental conditions. The gene content in a given population of a species often differs, quantitatively or qualitatively or both, from that of another population of the same species in another locality, and frequently no explanation of the different gene contents can be given on the basis of Natural Selection. Two distinct variations of seed-coat occur in *Silene maritima*. Figures from analyses of a large number of wild populations indicate very different percentage occurrences in populations in the British Isles (Marsden-Jones and Turrill, 1928-36).

The degree and kind of plasticity and of adaptability varies greatly from species to species, and even from genotype to genotype. This has been well shown by the British Ecological Society's Transplant Experiments at Potterne (Marsden-Jones and Turrill, 1930, 1933, 1935). Susceptibility to a disease limits the distribution of some genotypes, as is well shown by races of *Silene Cucubalus* grown at Kew and Potterne from Balkan Peninsular seeds (Marsden-Jones and Turrill, 1928-36).

Natural Selection acting on a given population under one set of environmental conditions acts by reducing the otherwise possible polymorphism. It is an agent which favours the formation and maintenance of distinct species in distinct environments. Its sieving action is excellently illustrated by the distribution of *Silene alpina* and *Silene Cucubalus*. In the French Alps *S. alpina* occurs on screes. *S. Cucubalus* penetrates practically continuously from the lowlands as a woodland-edge plant and as a constituent of the hay meadows. One typical instance may be given of their distribution. Below the Meije, opposite La Grave, the valley of the Romanche has meadows with *Silene Cucubalus* as one of the dominant constituents. These meadows are cut in July when the seeds of the *Silene* are ripe. To watch the seeds shaken out from the capsules as the hay is carried is a clear demonstration that selection—if not fully natural—has played a part in the distribution of the plant. The tall erect habit of *S. Cucubalus* enables it to compete successfully with other tall herbage. On an outcrop of schists the vegetation is very open, but the chief species is *Silene alpina* with low habit of growth, small leaves, and 1-flowered inflorescence. The two distinct habitats are separated only by a foot-path but *S. Cucubalus* never invades the territory of *S. alpina* and the latter with its low habit of growth could not survive competition with the tall, close herbage of the meadows. A few hybrids were found inter-

mediate in habit characters, but only in intermediate habitats. Experiments have shown that the two species can cross reciprocally, and they do cross naturally, as is shown by the occurrence of hybrid plants in the wild and by analysis of plants raised in considerable numbers at Kew from wild seed. Seeds collected 50 to 100 m above the meadows yielded mainly pure *S. alpina*, but 12% of hybrids. Yet the two natural populations of adult plants are specifically quite pure and the two species are certainly not under the existing conditions, amalgamating to one more polymorphic species. The physiographic, edaphic, and biotic conditions of the two habitats act as a sieve. This illustration is important for one other matter. *S. Cucubalus* and *S. alpina* are very closely related species though they differ, at least statistically, in a number of characters of which habit is an important one, and it has been said that it has never been shown (at least for animals) that "the structural characters by which closely allied species were distinguished had any adaptive relation to the environment in which the animals were living" (Elton, 1930). Further, we have here a good example of the survival value of what is usually described as *habit*. Studies on various floras have led to the conclusion that habit is most important so far as Natural Selection is concerned with the distribution of flowering plants.

Many existing distributions are largely due to changed environments. In the Balkan Peninsula some of the largest genera are characteristic members of the vegetation of deforested hill and mountain slopes. Examples are *Verbascum*, *Thymus*, *Silene*, and *Dianthus*. The destruction of forests has enabled species within these genera to spread, to meet, and so to hybridize with the production of a wealth of new phenotypes which are the bane of the taxonomist but make material for Natural Selection (Turrill, 1929).

When environmental conditions change in the same direction contemporaneously over a large part of the area of a species the chances are increased of selection of a new population, or of a new species essentially different from the old, or from that found in other parts of the original species population. Such *hamagenesis* is a very probable origin for certain pairs or groups of vicarious species, *i.e.*, species with contiguous but little overlapping areas of distribution. This type of distribution is fairly frequent in the Balkan Peninsula, and is well exemplified in *Abies*, *Chionodoxa*, *Fagus*, *Gentiana*, *Hypochaeris*, and *Crepis* (Turrill, 1929).

Natural Selection seems sometimes to act as a sieve concurrently with the migration of a polymorphic species into new environments and micro-species or subspecies not very clearly delimited from one another may result. The *Ajuga chia-chamaepitys* group extending from the Eastern

Mediterranean Basin westwards and north-westwards has lost its perennial—almost woody—habit and its large flowers (Turrill, 1934). On the other hand, it has not been possible to suggest any influence of Natural Selection to account for the distribution of the variations of *Glaucium flavum* s.l. (Turrill, 1933), or for the marked fruit variations in *Clypeola Jonthlaspi* (Chaytor and Turrill, 1935).

The importance of the best possible taxonomic knowledge of any group as a basis for the study of Natural Selection should be obvious, but it may well be that some examples of distribution cannot yet be interpreted by its action because of incorrect or inadequate classification of the phenotypes concerned.

Dr. T. J. JENKIN—*Natural Selection in Relation to the Grasses*—Large populations of normally wind-pollinated grass plants, derived from many different sources and habitats, at once reveal the great variability that exists within the species.

During the last fifteen years at the Welsh Plant Breeding Station, I have studied about 75,000 different plants of *Lolium perenne*. Most of them have been grown from seed, and the seedling stages have been studied in the first year under conditions where the plants are free from competition. In the second year, under the same conditions, when the plants are at full maturity, their seasonal behaviour and development are studied.

From our present point of view, the results may briefly be stated as follows: (1) Individual plants, even those derived from a relatively stable habitat, differ one from another in phenotype. (2) This variation, although quite definite, is usually within a relatively narrow range. (3) Plants from the same habitat, which are not very dissimilar phenotypically, may differ widely genetically. (4) Plants from different habitats conform to different general types. (5) In a large population representing many sources of origin, an infinite number of grades and intergrades occur, but the extremes do not suggest incipient species. (6) Very extreme types when intercrossed yield fully fertile progeny.

Within the species, therefore, great variability occurs, and it would be interesting to trace what is known of the history of the species in relation to type and distribution. All that I wish to say at present is, that it is unlikely that our cultivated type is directly derived from the old pasture types. Definite selection under semi-natural or highly artificial conditions has resulted in a divergence of type.

Of the other *Lolium* species, I have only seriously studied intra-specific variation in *L. italicum*. This shows a wide range which may, at least

partly, be due to natural crossing with *L. perenne*. The two species agree in being normally cross-fertilized, but they disagree in morphology, and the capacity for vegetative regeneration after ripening seed is weaker in *L. italicum* than in *L. perenne*. Intercrossed artificially, they yield F_1 hybrids of high fertility, while a wide range of types is obtained in the F_2 generation. Despite high fertility, the F_1 hybrids show slight irregularities in meiosis.

L. rigidum is also normally cross-fertilized, and has many morphological features in common with *L. perenne*; but it is a strict annual in the sense that it has no capacity for vegetative perpetuation after ripening seed. It can easily be intercrossed with *L. perenne*, and the F_1 hybrids are highly fertile, but again there are slight irregularities at meiosis.

L. temulentum, *L. remotum*, and *L. loliaceus* agree with *L. rigidum* in being strict annuals, but they differ from it morphologically and in being normally self-fertilized. *L. temulentum* and *L. remotum* can easily be intercrossed, and they yield seed of good germinating capacity, while the seedlings and mature plants are of normal vigour. The fertility of the F_1 hybrids has not yet been studied, but the plants are in every respect so normal that I am expecting to find that they are fully fertile.* If this proves to be the case, the obvious suggestion will be that *L. temulentum* and *L. remotum* are merely genotypes of the same species, that have become separated by purely mechanical means through association with crops which are different in size of grain or seed.

L. temulentum and *L. remotum* have been intercrossed with *L. perenne*, but only the *perenne-temulentum* hybrids have yet been studied. These F_1 hybrids are male-sterile, but can be used as female parents in backcrosses to either parent species. Meiosis in the F_1 hybrids is less regular than in the *perenne-italicum* and *perenne-rigidum* hybrids.

L. temulentum again has been successfully intercrossed with *L. loliaceus*. The young seedlings were by no means excessively weak, but in later growth stages they are making very poor headway. The difficulty appears to be to some extent mechanical, the leaves and inflorescences failing to expand normally.

When we come to review the genus as a whole on the basis of these observations, together with what we know concerning the distribution and habitat requirements of the various types and species, we have reason to

[* *Note added in proof*, 1 July, 1936—This expectation has not been fulfilled, the F_1 hybrids proving to be highly, if not absolutely, male-sterile. Mr. P. T. Thomas, B.Sc., informs me that in meiosis there is normal pairing of the chromosomes, and that he has observed no lagging chromosomes. Failure, therefore, occurs at a late stage in pollen grain formation.]

believe that they are all phylogenetically related. At present, however, we find material differences between the species not only in morphology but also in distribution. Three of the species, *italicum*, *temulentum*, and *remotum*, have been, and are being, preserved owing to the direct action of man—intentionally in the case of *italicum*; unwillingly in the case of the other two. It is difficult to determine whether, in the absence of any interference by man, any one of these could persist in nature. *L. loliaceus*, on the other hand, appears to be able to hold its own in nature under certain conditions. This is also true of *L. rigidum*, but in recent years its territory has been greatly expanded, particularly in Australia, through the action of man. *L. perenne* persists under semi-natural conditions mainly, but although it shows great variation in plant type, and great virility, it is really aggressive only under rather well-defined conditions.

All the species have remained diploids, so that the genus owes nothing to polyploidy. Fundamental changes, probably in cell physiology or chromosome morphology, have, however, produced distinct types which we call species, but although there is some overlapping, they occur mainly in different areas. In general, the annuals are more southerly in their distribution than the perennial *L. perenne*.

The genus *Festuca* shows an entirely different state of affairs, except that again a variety of types occurs within each species. I have studied mainly *Festuca* species belonging to the two groups *Ovinae* and *Bovinae*. In each, various polyploids occur. In the *Ovinae* we have, for instance, the diploid *F. capillata*, the tetraploid *F. ovina*, hexaploids and octoploids of *F. rubra*, as well as higher polyploids. Of these, I have only inter-crossed *F. ovina* and *F. rubra*. The F_1 hybrids are male-sterile, but can be back-crossed to both parent species.

In the *Bovinae* I have studied the diploid *F. pratensis* and the two distinct hexaploids *F. arundinacea* and *F. gigantea*. The F_1 hybrids from each of the three possible crosses are male-sterile.

A connexion between the *Ovinae* and the *Bovinae* has also been found in the hybrids between the hexaploids *F. rubra* and *F. arundinacea* produced artificially.

The ease with which F_1 hybrids can be produced artificially differs greatly from one pair to another, but on the assumption that compatibility of such a degree that the production of F_1 hybrids is possible signifies phylogenetic relationship, the fescues, despite the morphological differences between the *Ovinae* and the *Bovinae*, appear to form a natural group.

Each species, however, has a distinct range of distribution. The diploids, although less aggressive than the diploid *Lolium perenne*, still hold their own. In the *Ovinae*, *F. ovina* is mainly outside the normal

territory of the diploid *F. capillata*, and the tetraploid *F. ovina* and the hexaploid *F. rubra* only barely overlap. Similarly, the diploid *F. pratensis*, and the hexaploid *F. arundinacea* rarely occur in close association one with another, while the other hexaploid, *F. gigantea*, is definitely more shade-loving than either, and its high self-fertility is particularly useful where there is relatively little wind.

While, therefore, *Lolium* has extended its territory without changes in chromosome numbers, *Festuca* has depended very largely upon polyploidy as a means of expansion. In both cases, the different types occupy different situations, and compete less with one another than with other grass species.

When, further, we study the two genera *Lolium* and *Festuca* together, I find that *L. perenne* can be successfully intercrossed to yield established F_1 hybrids with each of the following species: *F. rubra* (in the group *Ovinae*), and *F. pratensis*, *F. arundinacea*, and *F. gigantea* (in the *Bovinae*).

On the assumption already made, this means that *Lolium* is again phylogenetically related to members of both the *Ovinae* and *Bovinae* groups of *Festuca*, and still we do not find serious competition between the species except in the two diploids, *L. perenne* and *F. pratensis*.

It would, therefore, appear that where distinct species have been successfully produced and preserved, they must occupy different situations from those in which the types from which they are derived normally exist. The new *Festuca* polyploids have not replaced the diploids, but have found suitable habitats outside the normal range of the diploids. In these cases we know that the changes have involved fundamental changes in the cell, and although polyploidy is not met with in *Lolium*, changes in the cell itself are again suggested by irregular meiosis in F_1 hybrids.

The impression gained through these studies, therefore, is, that Natural Selection certainly aids the preservation of certain new types when they have been produced, but this happens at the expense of species belonging to other genera.

Returning to *Lolium perenne* with its ecotypes and intergradations, we find no evidence of such a fundamental change as appears to be required for the production of new species, unless we regard *L. perenne* as a recent offshoot of *L. rigidum* or *vice versa*. Whether the extreme types of *L. perenne* are tending towards new species it is impossible to say. The evidence available suggests that even the extreme variations are due to normal gene differences. The isolated position of the *L. perenne* area in south-west Norway appears to be the most favourable place for the production and perpetuation of fundamental changes, but although very

distinct in many ways from our *L. perenne* types the Norwegian still interbreeds freely with British types when the conditions are rendered favourable.

I am indebted to graduate research students, Messrs. Evans, Sethi, Peto, and Thomas for the cytological data.

PROFESSOR R. RUGGLES GATES, F.R.S.—*Mutations and Natural Selection*—Since Darwin's time our knowledge of variation has enormously increased, but actual observations of natural selection at work are extremely meagre, and there remains wide divergence of opinion regarding the role played by natural selection. Mutations are generally regarded as the raw materials on which selection may act with more or less effectiveness.

It appears that in mathematical theories of selection a distinction should be drawn between the whole population of individuals comprising the species, and the effective range of interbreeding of any one individual. In plants, the effective intercrossing population will be limited by the fact that most plants are both stationary and hermaphrodite, but it will be extended by the visiting ranges of insects or by the pollen-carrying range of the wind.

The study of native floras discloses many cases where an apparent mutation has spread without aid from natural selection, or even in the face of selection. An instance is *Saxifraga Virginiensis* var. *pentadecandra*, which was formerly abundant in Essex Co., Massachusetts. The absence of petals in this form can hardly be regarded as anything but a disadvantage. *Drosera rotundifolia* var. *comosa* is a teratological form occupying half an acre in a sphagnous bog in the Gaspé peninsula, Quebec, to the exclusion of the type, which is abundant around the bog. Here selection appears to have favoured this dwarf form under bog conditions. *Maianthemum canadense* in North America is generally glabrous, but a heavily hirsute variety is found in an arid region of South Dakota. This is probably a mutation which enables the species to extend into this arid area. *Epilobium glandulosum* has given rise, on the tidal mud flats of the St. Lawrence estuary, to *E. ecomosum* Fern., which has lost the coma from its seeds, and whose seeds are grey and ridged instead of brown and minutely pebbled, the rest of the plant remaining essentially unchanged. The new characters appear to be adaptive, at least in part, to the peculiar conditions. Crossing experiments would be of great interest, and should give some indication of the steps by which this differentiation has taken place.

In the genus *Antirrhinum*, Baur found by crossing that the various

European species were distinguished by simple Mendelian characters. Kristofferson intercrossed seven species of *Malva* and finds that the specific characters all show Mendelian segregation. In such features as flower-size and number of carpels multifactorial segregation is shown, and the same is true of flower-size in various other genera. Several specific differences in *Malva* were found to be due to single factors. *M. oxyloba* Boiss. differs from *M. parviflora* L. in having sharply serrate leaves and long-pointed sepals, as well as in being shorter with a more slender stem. All these differences behave as a unit, *M. oxyloba* being dominant, and the size differences probably result from its smaller leaf surface. *M. oxyloba* is found in Cyprus, Palestine, and Syria, while *M. parviflora* is found not only here but also in Europe and from India across North Africa. Whichever is the parent form, *M. oxyloba* has spread over quite a wide area, although natural selection must favour its stronger rival.

Modern cytological work shows that many new genera and species have been produced in connexion with tetraploidy, amphidiploidy, chromosome duplication, and interchange. These internal nuclear changes are accounted for by the nature of the cell mechanisms involved. After such new types have appeared, natural selection may aid their spread, as in the case of the amphidiploid *Spartina Townsendii*. Hagerup has found evidence that polyploid species of plants are adapted to more extreme conditions than their diploid relatives, both in the Arctic and in the Sahara. Tischler estimates that 44% of the Angiosperm flora of Schleswig-Holstein are polyploid species; while Rohweder has recently concluded that lime in the soil-water favours the presence of polyploids as against diploids.

The conception of parallel mutations, which dates from 1912, has become of increasing importance. A single probable case from flowering plants may be cited. *Betula nana* var. *Michauxii* in Newfoundland differs from the type in having the bracts of the female flowers entire instead of 3-lobed. *B. alba* var. *elobata* Fern., from Mt. Albert, Gaspé, differs from the species in the same respect. This character can have no selective value, either positive or negative, and it is typical of innumerable characters differentiating plant species.

Innumerable parallel characters can now be recognized in all groups throughout the plant and animal kingdoms, from the Cyanophyceae to the Primates, and they appear to have arisen as mutations. Some structures have been perfected as adaptations through a longer or shorter series of stages, as in the case of elaters, which have arisen several times independently not only in liverworts but also in Mycetozoa. In animals,

the longer parallel series are usually concerned with adaptations to a different medium or habitat, and natural selection has presumably been at work. This explanation cannot always be applied, however, for the Cetacea and Edentata, although adapted to wholly different conditions, yet show several striking parallelisms of specialization which their common ancestor could not have had.

The problem of the limits of parallelism has become of fundamental importance, for there must be limits if evolution as we understand it has taken place. In any case, the classical conception of a branching tree to represent the phylogeny of a group, with the youngest twigs representing the modern species, requires alteration. Darwin's conception of modification with divergence must still apply widely, but parallelisms require a different method of representation, and the term convergent evolution is often misleading. It appears that the phylogeny of most groups would be better represented by one of the strangling figs, which send out numerous parallel roots from their branches, these roots frequently coalescing later with each other or with other branches.

It may be concluded that while natural selection appears to have played an important part in some of the longer parallel series, it cannot have been a factor in all. Modern species show innumerable parallel mutations, a few of which have spread because they were adapted to a peculiar habitat, while large numbers have succeeded in spreading, at least to some distance, without any aid from natural selection or even in spite of it.

Professor R. A. FISHER, F.R.S.—*The Measurement of Selective Intensity*—Theories of Evolution are of two kinds, those that, in Professor Watson's words, "are explanations primarily of adaptation and only secondarily of the origin of species", and those which fail to account for adaptation. To the first class belongs the theory of the inheritance of acquired adaptations, commonly called Lamarckism, and the theory of the natural selection of innate adaptations. For these two theories evolution is progressive adaptation and consists in nothing else. The production of differences recognizable by systematists is a secondary by-product, produced incidentally in the process of becoming better adapted.

I do not know whether, at the present date, anyone is prepared to advocate a theory of the development of living organisms, which ignores the necessity of explaining adaptations, in the wide sense of that term. Such theories also are of two kinds. It has been proposed that animals evolve by reason of something which may be called an inner urge, implanted in their primordial ancestors, which causes a progressive change

of form along a predetermined course, undeterred by any difference in the death-rate or birth-rate that may be set up. It has been proposed, alternatively, that the environment, picturesquely renamed the landscape, governs the course of evolutionary change, much as the field of force determines the trajectory of a comet. One might think that these two theories between them exhausted the narrow possibilities open to inventors of theories of non-adaptive evolution, but the example of the so-called mutation theory, once popular among geneticists, shows that this is not so. The explanatory content of a theory of evolution only reaches its absolute zero with the mutation theory. Organisms evolve, that is to say, heritable changes take place in them, because they mutate—because unexplained heritable changes take place.

I submit that the omission of this group of theories to explain adaptations is, from the most elementary point of view, an intolerable one; for they fail totally to explain the functioning either of the entire organism or of any of its parts. Palaeontologists, impressed by the disappearance of some imposing creature, as they pass from one stratum to the next, have sometimes rashly surmised that this was evidence that they had become less well adapted to the current conditions. But even they will admit, if their subjects had eyes, that they were functional organs, not inoperative as optical and sensory systems; that their instincts impelled them to catch their prey, or to browse on their forage, and to carry out the necessary functions of their reproductive cycle; that their nervous systems did convey afferent and efferent impulses; and, perhaps, that their digestive systems were in good working order. This simple list implies, I suppose, some hundreds of nicely adjusted adaptations. Good work, one might say, for the primordial "urge" to have foreseen, or for the moulding finger of the "landscape" to have created.

For *rational* systems of evolution, that is for theories which make at least the most familiar facts intelligible to the reason, we must turn to those that make progressive adaptation the driving force of the process. Lamarckism was first in the field, and the reasons for its abandonment are familiar. But it is worth considering why, during the three-quarters of a century for which Darwin's views have been known, so many biologists have felt, and raised, objections to them.

I believe the explanation must be more temperamental than intellectual. Darwin's work reached a wide public. His views, or something like them, were supposedly familiar to all educated men. That being recognized, few scientific specialists, specialists at least in biology, could suppose that *they* had anything to learn from his works. Even the "Origin", when read at all, would seem to have been read cursorily, without the

expectation of learning anything from it. It always seems more knowledgeable to expound the *latest* views, however flimsy.

Then, fireworks look much brighter against a *black* background. Biologists, exploring a vast field, for the greater part of which the public care nothing, have, I think, sometimes been tempted by the illusion that their valuable findings have a bearing, which they really lack, on evolutionary doctrine, in which, for a time, the public really were interested. That this interest has since largely evaporated must, I think, be principally due to the intellectual frivolity of the discussions on evolution during the past 40 years, which it has been customary for biologists to stage. The old arguments on which the whole subject still rests have been neither answered nor furthered, and Darwinism has been too often misrepresented merely to supply a black background against which to exhibit the brilliance of modern advances.

In places some of the black paint seems to have stuck. In his second paragraph Professor Watson says that Darwin's theory "rests on the assumption that *the* differences between individual members of a species are heritable". Should he not have said "some of the differences"? For that is sufficient for natural selection. Then the alleged "assumption" is reduced to a mere rejection of the postulate that all members of the same species are genotypically identical. I believe no one asserts this postulate; and that it is known to be untrue of all species, without exception, which have been investigated. It is a peculiarity of Darwin's theory, that it relies only on causes demonstrable independently of their evolutionary effects.

In plants at least, Turesson's results in Sweden are abundant and conclusive in showing that different ecotypes do very generally differ genetically. Agricultural experience is also wide and uniform in finding obvious differences in the adaptation of different varieties of cultivated plants to different climates. Professor Watson suggests that similar knowledge is lacking in the case of animals.

Professor Watson also seems to have real doubts of the existence of differential mortality between different genotypes in natural populations. Is he really tempted by the inconceivable assumption that the net measure of fitness, whether thought of in terms of death or reproduction, for it must involve both, is *identical* in all the environmental situations which occur, even for any two of the thousands or millions of genotypes which most species must have? Or is he only providing a cue for discussion? I will try to answer his point.

Some years ago I suggested that in polymorphic species we have natural markers maintained in equilibrium by a balance of selective agencies.

The equilibrium is stable only if the heterozygotes have an advantage over both homozygotes, but the fact of selective balance allows, in these cases, selective intensities to occur greater than can exist elsewhere, that is, unbalanced, merely by reason of the rapidity with which any species subjected to them would be transformed. The selective intensities may be measured by the known crossover frequencies against which they are balanced. I had some correspondence with Dr. Nabours of Manhattan, Kansas, who was able to organize an extensive collecting trip in Texas and Mexico with a view to ascertaining the frequencies with which different gene combinations, recognizable, on the basis of Dr. Nabours's genetic work, from the colour pattern, were found among a number of species of grouse-locust. I must admit that I believe neither Dr. Nabours nor I have published anything about it. He sent me quantities of illuminating data, and I sent him very lengthy dissertations about them. I am confident that Dr. Nabours will forgive me for mentioning the matter now, for certainly every one of the species *Acrydium arenosum*, *Paratettix texanus*, *Paratettix cucullatus*, *Apotettix eurycephalus*, for which large samples were collected, showed unmistakable evidence of differential survival among the genotypes in their wild habitats. In most cases the differential elimination was sufficiently moderate for it to have been due either to death only in the period between the formation of the zygote and the time of capture, or in the other half of the cycle between the time of capture and the formation of the next generation, when both differential mortality and differential fertility would be effective. In one case, I remember in *Acrydium arenosum*, no amount of differential fertility, even complete sterility, would suffice to explain the disparity in numbers observed, for crossing over alone would produce more than were observed. In fact, the figures could only be explained by the differential elimination down to about 40% of one genotype mahogany *My* without white line *W*, compared with its competitors. The low viability of this type in nature was further confirmed by breeding experiments in which an even lower viability was indicated in culture.

That, however, is only an extreme case out of many, and I must emphasize again that only in balanced systems are selective intensities of this order to be expected. The selective intensities effective in evolutionary change are, I believe, more likely of the order of 1% to a tenth of 1% in each generation. Breeders of *Drosophila*, if they set their hearts on it, could, I think, just manage to breed enough flies to detect differences in viability and fertility of these magnitudes. I do not know the series of writers which Professor Watson refers to as having tried to demonstrate the existence of selection without success. I would suggest, however,

that it is no evidence against the existence of the planet Pluto that he cannot be seen with even quite a good opera-glass. Negative evidence is worthless if we do not know both the magnitude of the theoretical effect to be observed and of our errors of observation.

C. DIVER—*The Problem of Closely Related Species and the Distribution of their Populations*—The degree to which Natural Selection is responsible for the production of adaptations and the origin of species is still a matter which must be discussed largely on indirect evidence. Recent genetical and mathematical work has greatly increased the reasonableness of the theory, but it has mainly been directed to demonstrating that Natural Selection could operate and not that it does operate. The lack of direct evidence is due partly to the difficulty of detecting in natural conditions the small amount of selection that would theoretically be efficient and the superficially insignificant characters that are probably the most important material for its operation, and partly to the neglect of direct studies of natural populations. Such direct work as had been done in the field brings further difficulties to light.

Adaptations for the purposes of this brief argument may broadly be grouped as: (1) those that appear to be reactions to external stimuli including (a) protective adaptations against predatory attack, and (b) response to more general ecological conditions; (2) those that might arise from internal stimuli.

I have made some attempts to determine whether there is the requisite selective death-rate in the attacks of birds upon snails. The figures collected looked in themselves quite hopeful; but enquiry into the nature of the populations from which they were drawn at once showed that no proper use could be made of such data until a great deal more was known about the structure, distribution, and behaviour of natural populations generally. The South Haven Peninsula survey was undertaken in the hope of supplying this information.

Close protective adaptations are often so amazing that there is a serious danger of their obscuring the picture; and perhaps the comparatively few species that show them are not always put in their true perspective among the great mass that do not. Whatever way one looks at it, close adaptation is in effect a limitation, and in the long run limitations are usually disadvantageous rather than the reverse. This becomes more obvious when internal adaptations are considered, and even more so in regard to ecological adaptations.

The internal stimuli considered here are mutations in the broad sense;

and the limiting factor obviously is whether the change is of such an order that the organism can, or cannot, still remain an effective functioning whole. A gross change that passes this limit will be selected out because, *ex hypothesi*, the organism will not live to sexual maturity. The distance of this limit from normality for any particular organism should surely be a rough measure of the organism's capability to overcome by small readjustments in its general scheme the upset produced by the mutation, *i.e.*, a measure of its preservation of adaptability and an inverse measure of its degree of specialization.

From this it follows that those species which retain a wider range of internal adaptability will be capable of showing in their populations a wider range of variability. Abundant or common species often show a greater variability than those less common. If relative abundance within a group of allied organisms is taken as a rough measure of success, this means that the successful species are those that preserve a wide adaptability.

Here is the dilemma. Natural Selection seems the only rational explanation of adaptations, and it has been shown that the pressure of selection must finally produce the closest adaptation. The difficulty lies in invoking at the same time the same explanation for the preservation of wide general adaptability. If selection favours, as it should, the advantageous capability to vary, then its pressure upon the products of this variability must be removed if the attribute is to remain advantageous. Theoretically this can be done, *e.g.*, by making the quite gratuitous assumption that the variability displayed by abundant or successful species is maintained by high mutation rates. Without any direct evidence this type of explanation is not very satisfying.

An examination of groups of closely related species shows, first, that most species are distributed in small discontinuous population units or colonies. (Incidentally Wright has shown that this is the most favourable distribution for the operation of selection.) Secondly, that such species frequently live together in mixed colonies, *i.e.*, in similar if not identical ecological conditions. Thirdly, that species within such a group differ from each other in the range of habitat types that they can effectively occupy. For example, if a series of different habitats be arranged from dry to wet, species A may be confined to a narrow range at the damper end of the scale, while B may have its optimum in the same place but a wider spread on one or both sides. Methods are now being developed at Studland of measuring the degrees of tolerance thus expressed. A species to be ecologically successful (in terms of commonness or abundance) requires, generally speaking, a wide "tolerance range". Again,

then, wide adaptability and not close adaptation must be produced and preserved if a species is to become and remain successful.

The detailed study of such species also provides facts that bear on the problem of their origin. The two common snails *Helix hortensis* and *H. nemoralis* are very closely allied species with, as would be expected, almost a completely parallel range of variability. They have a wide overlap in tolerance range and in geographical distribution. They frequently live together in mixed colonies. They are undoubtedly good species separated by clear genital differences. They can be made to produce fertile hybrids, but it is unlikely that they do so in nature to any appreciable extent. Theoretically, selection must have favoured the establishment of the new genital characters in one group of the common stock and so split it off, while at the same time leaving *both* derivative groups about equally common and successful. A chance mechanical isolation seems a much more likely explanation of this split.

Again, three very close species of the genus *Crambus* have similar tolerance ranges, but that of the common species is much wider than those of the two local species. These latter within their limited habitats are successful in the sense that though very local they may be abundant where they occur. The known differences between them are very small, and all three produce good colonies in the same spot under apparently the same ecological conditions. Here isolation may have been brought about by variations in the peak period of sexual maturity.

The Myrmicine genus *Strongylognathus* has apparently developed a species on this side of the Channel, though it and its close relative on the Continent both live in the nests of the same species of *Tetramorium*.

Similar facts could be given for the plant genus *Cerastium*. The large array of species in *Rubus*, *Rosa*, and *Hieracium* and the possible causes of their origin are well known.

To summarize: apart from the elimination of organisms that try to transgress the limits of their internal adaptability, it seems possible that the necessary selective death-rate may be demonstrated for certain types of adaptation, and probable that close adaptations are the product of selection pressure limiting and perhaps directing the natural expression of variability. But the conditions in which this pressure is exerted are less common than is generally supposed, and their onset suggests that the species is beginning to lose ground.

With regard to the origin of species, it has been shown that a type of evolution could be brought about by Natural Selection; but the more deeply the facts relating to closely allied species are investigated, the less likely does it appear to become that the evolution that has taken place is

of this particular type; and the more likely is it that species formation may be brought about by a number of different causes of which selection might possibly be one.

Professor G. D. HALE CARPENTER—*The Facts of Mimicry Still Require Natural Selection for their Explanation*—Mimicry is the term applied to the deceptive resemblance whereby one animal is mistaken for another by its natural enemy. The phenomena are similar in resemblance to a stick or stone: the animal escapes being eaten because it resembles either an object which the enemy knows by previous experience to be distasteful, or one which is of no interest from the point of view of food value. Natural Selection is the only theory which accounts for both sets of cases.

The following facts require Natural Selection for their explanation:

1—The aposematic models have characters not found in species highly appreciated for food: conspicuous red and yellow colours in simple, easily recognized, patterns; and habits increasing conspicuousness. These visible characters are associated with unusual resistance to injury, enabling such an insect to be handled, examined, and even tasted, without serious damage. Also, the possession of spines, stings, etc., and emission of offensive odours or bubbles of acrid froth. The nocturnal conspicuousness of a glow-worm is associated, as would be expected, with distastefulness.

2—There is experimental and observational evidence that aposematic species are not taken as food when choice is possible.

3—Education of the enemy is assisted by the similar appearance of many distasteful species, often widely apart in phylogeny. Each gains by the lesson learnt by the enemy from the other. Such Müllerian resemblances are commoner than the Batesian resemblance of a highly edible to a less edible species.

4—This is as it should be, for too abundant deceptive mimicry would defeat itself.

5—There is no such thing as absolute inedibility. A species is more edible than some, less edible than others. A species in one form in one part of its area may serve as model; in another part it comes under the influence of a predominant species and becomes a mimic.

6—The defensive value of aposematic coloration depends upon abundance of other food. Mimicry is best exemplified in the presence of abundance of life, and may be shown only in the wet-season form of a species.

7—Mimicry deceives the artist but not the anatomist. Natural Selection is only required to produce a deceptive superficial appearance to

keep the enemy at a distance: an exception is afforded by deep-seated neuro-muscular changes involved by a change of habits when such change aids the mimetic effect.

8—Natural Selection characteristically effects the same ends by quite different adaptations. A mimetic resemblance to one model may be attained by most diverse means.

9—Polymorphism in a mimic is not to be explained as mere differences in appearance such as may occur in any species. This would not explain why the forms of a polymorphic mimic should almost always resemble some aposematic species. Polymorphism enables a mimic to profit by resembling different models in different parts of its range, or in the same part. A mimetic species, unable to follow changes undergone by its model in another area, bears a resemblance to another species of which the appearance is within its power to copy.

10—The niceties of geographical distribution of models and mimics controvert any explanation based on chance.

11—The polymorphic butterfly *Pseudacraea eurytus* (L.) follows closely in appearance the predominant species of *Bematistes* (= *Planema*) which serve as models in any particular locality. If in one locality the models greatly decrease in proportionate number their protective influence diminishes, the appearance of the *Pseudacraeas* is uncontrolled, and intermediates between the completely mimetic forms, usually extremely scarce in the field, survive. When the number of models in that locality again increases the proportion of non-mimetic intermediates in the *Pseudacraeas* falls to the usual very small figure.

12—Parallel mutation is not an explanation of mimicry: it fails to account for the case of *Papilio dardanus* Brown, of which most of the female forms mimic species of *Danainae*. But one form mimics an *Acraeina*, a species of *Bematistes*, yet it is linked with the others by transitional forms. The Pierine genus *Euterpe* has species mimicking *Papilionines*, but a slight change in the pattern in one species results in a *Heliconine* appearance, opening up a new series of models. Moreover, even in the case of *Hypolimnas dubius* Beauv., which has two forms mimicking different species of *Danaines*, the details upon which resemblance depends are not the same. The difference between the forms of *dubius* is, however, controlled by a single gene. And how can resemblance of an insect to a leaf, completely analogous to the resemblance of one insect to another, be explained by parallel mutation?

13—Nor can mimicry be explained by environmental influences. Beetles of the family *Lycidae* all the world over are resembled by insects of many different orders: they have been proved to be highly distaste-

ful. But the appearance of the *adult* insects is prepared during larval stages passed under conditions of food and environment as different as possible.

14—If mimetic resemblances are due to factors other than Natural Selection why are they only to *conspicuous* objects? Dull coloured species may show resemblance to surroundings but do not mimic each other.

15—According to the theory of Natural Selection, the rarity of true Batesian mimicry is explicable in the same way as polymorphism among dead leaf butterflies: the risk to an edible individual would be too great if too many resembled the same object. The explanation is complementary to that for Müllerian resemblance between distasteful species: this benefits both insect and enemy by *reducing* the work to be done by the latter in learning his patterns, while multiplicity of dead-leaf patterns *increases* the work to be done in finding food and diminishes the risk to each individual pattern, to the advantage of the species as a whole.

Professor J. B. S. HALDANE, F.R.S.—*Primary and Secondary Effects of Natural Selection*—Natural Selection may be defined as the elimination from a genetically mixed population of the less fit genotype. The fitness of a genotype is measured by the mean number of progeny left by its members, subject to certain conventions. Clearly if natural selection is not counteracted by some other process it will lead to a change in the population. And since the difference between a number of related species has been at least in part analysed in terms of genes, this change may be equated with evolutionary change. However, in most cases Natural Selection seems to be pretty exactly balanced by mutation. This does not prevent us from studying it.

Evolution is generally a very slow process, giving little detectable result in 20,000 generations. Differences of fitness sufficient to account for changes taking place at such low rates must often be too small to detect. Thus if a dominant gene increases the fitness of its carriers by one part in 1000, its frequency in a random mating population will increase from 0.001% to 99% in 23,400 generations. Now if this difference of fitness affected viability we should need a population of at least sixteen million to detect it even with the degree of certainty afforded by a deviation equal to twice its standard error. If it affected viability we should have to count the progenies of at least sixteen million individuals. If a difference of fitness were determined by the compresence of several genes it might be much larger and still spread at the above slow rate. But it would be very hard to detect.

Actually the reality of Natural Selection can be demonstrated easily enough. It is known that new genes are constantly arising by mutation, and that most such genes lower fitness, at least when acting one at a time. If they did not do so they would already have spread through the species. Hence in every species there is an equilibrium between mutation and selection, and except when a species is changing very quickly the main function of selection must be to keep it constant.

Both dominant and sex-linked recessive mutants are selected out very rapidly. But autosomal recessives are only eliminated in homozygotes which contain two of them. In a species which is not closely inbred harmful autosomal recessive genes are shielded from selection by their normal allelomorphs. This is theoretically obvious, and has been verified by Gordon in a field experiment involving the release of 18,000 flies.

Hence the inbreeding of the progeny of members of an apparently uniform outbred wild population should reveal many autosomal recessives, but few other genes. This has been verified in four *Drosophila* species by the authors cited by Timoféeff-Ressowsky in his communication, by Jenkin and Williams in a variety of pasture plants, and by Spooner in *Gammarus chevreuxii*. Only in one *Drosophila* population were genes of other types found with appreciable frequency.

The forms so obtained are similar to, and often seem to be identical with, laboratory mutants. Some, *e.g.*, those with dark body colour and rotated genitalia, may represent atavisms or possible future evolutionary steps. Others seem to be off any probable evolutionary path. If their appearance were due to the change of environment, the great rarity of dominant and sex-linked genes would be unintelligible. The facts fully confirm Tschetwerikoff's theory as to the balance between mutation and selection.

In man the analysis of epiloia by Penrose, and of haemophilia, shows that the genes for these two conditions are constantly appearing by mutation with a frequency of about 10^{-5} per chromosome per life-cycle, and are quickly eliminated by Natural Selection. Fisher has already dealt with the stabilizing influence of selection in polymorphic species.

While the stabilizing effect of selection is readily detected, visible evolutionary effects can only be expected in response to drastic and sudden changes of environment. Unfortunately, where such changes have been observed there is rarely any direct evidence that they have been due to selection. However, Harrison found evidence of a selective death-rate causing such a change in *Oporabia autumnata*. It must be remembered that morphological abnormalities have been shown to be physiologically advantageous in some environments. Hence no *a priori* assumptions as

to the effect of a given gene or fitness are a substitute for actual observation.

There is, however, fairly clear evidence of an evolutionary effect of selection for disease resistance, particularly in man. Pearson, Diels, and v. Verschuer, and others showed that resistance to tuberculosis is largely genetically determined, and the progressive diminution in European mortality from that disease may be explained as largely due to Natural Selection. An excellent opportunity for studying selection at work is afforded by the negro races which are at present being infected with it. It may well be found that the main pressure of evolutionary selection is on individuals susceptible to disease, for the simple reason that the microbic environment changes more rapidly than the climatic, metazoan, or metaphytan environments.

The fact that Natural Selection has a primary role in stabilizing species makes more plausible its secondary role in determining which evolutionary paths they will take among the very vast number which mutation renders possible. But we must go beyond Darwin, and admit that Natural Selection determines not only the normal type of a species, but also its range of variation.

For the present, field studies on Natural Selection are likely to reveal the primary stabilizing effect rather than the secondary evolutionary effect. Only when the primary effect is understood in considerable detail can we hope for concrete and quantitative data on the evolutionary effect.

Professor E. W. MACBRIDE, F.R.S.—I should not have intervened in this discussion by geneticists at all, had I not been invited by the Biological Secretary of the Royal Society to attend and express my views as an opponent of the Darwinian theory of evolution. Darwin's name is always associated with "Natural Selection", *i.e.*, the survival of only a few of the young produced by a pair of parents. This is an obvious fact; a single instance will stamp it in our memories. Of 100 smolts which descend a river on their way to the sea only five return as salmon. It was not, however, Darwin but Lamarck who first drew attention to this selection fifty years before the "Origin of Species" was written; Lamarck was, as Dr. Fisher has said, the first to propound a theory of evolution in the modern sense or "transformation", and Dr. Fisher went on to say that all knew why this theory had been rejected. Dr. Fisher is an eminent mathematician who has taken up biology late in life and I venture to say that he knows nothing at all about the reasons of the opposition to Lamarck.

Lamarck encountered bitter enmity because his opponents believed in the miraculous creation of the ancestors of every species. Afterwards, when the battle for "transformism" was in full swing Haeckel, founder of comparative embryology and fiery protagonist in the fight for evolution against special creation, hailed Lamarck's theory as a great support in the contest and dedicated his "History of Creation" to *Lamarck* and Darwin. It was the vogue of Weismann's theory which made Lamarck's views fall into disfavour and about Weismann, Przibram, one of our most eminent embryologists, has recently said "There are four fundamental dogmas in the Weismannian religion and every single one of them has been shown to be false".

But it is obvious that selection by itself can produce nothing new. Professor Haldane, with whose remarks I found myself unexpectedly largely in agreement, defined its function in the human race as the removal of people of defective constitution—and this is undoubtedly true. What Darwin's theory covertly assumes is that small heritable variations *in all directions*, good as well as bad, are always occurring for no assignable reason and that some of these may suit the environment better than did the type.

Do these variations really occur? This problem has been investigated by Johannsen for the bean, by Agar for a small Crustacean, and by Jennings for a Protozoon, and all these three obtained the same result. *Such small inheritable variations do not occur.* This, as Gessner has pointed out, has destroyed the whole basis of "evolution by natural selection". The geneticist seeks to escape from the dilemma by saying that although fluctuating variations are not heritable small mutations are. But this is reasoning in a circle, for what is a "small mutation" except a small "heritable variation"? Changing the name does not entitle us to fly in the face of the evidence. That what are called "sports" (or "mutations"), *i.e.*, gross deformations of the adult anatomy, are heritable is well known, but to invent the word "gene" for the unseen cause of the mutation adds nothing to our knowledge. Johannsen, who coined the word, expressed his deep regret for having done so and asserted that "genes" were superficial disturbances of the chromosomes and had nothing to do with true racial characters. This conclusion is borne out by a closer examination of mutations. Mohr, one of the greatest authorities on *Drosophila*, has compared the mutations of this fly to the colours of the spectrum. At the extreme red end he placed the modifying factors which have no visible results taken by themselves and which are only assumed to exist in order to make the numerical results fit genetic theories. As we pass towards the blue end we encounter mutations which produce

more and more effect on the visible anatomy and at the same time decrease the viability. In the blue we reach the semi-lethal mutations which are fatal and can only be reared as heterozygotes—ending up in the violet with the lethal mutations which destroy all the germ cells in which they occur. The same non-functional character and injurious effect on viability is seen in the mutations of *Gammarus* with which I have been familiar for the last ten years at Plymouth. These largely affect the eye, and it has recently been shown that the degeneration of the eye thus caused is totally different from the dwindling of the eye owing to disuse found in allied species adapted to darkness.

Lamarck's theory is that the environment has no direct effect on the animal; what alters the animal is its own functional activity stimulated by its response to the environment. That such changes take place in the lifetime of the individual is common knowledge; what is needed for the full proof of Lamarckism is to show that some small residue of this effect is passed over to the offspring. This proof has now been given, and will convince all those who are not resolutely determined to disbelieve in Lamarckism at all costs, and to catch at any straw rather than admit the truth of the evidence. Professor McDougall's experiments on the heritability of induced habit in rats have been carried on for nine years and repeated so as to meet all possible objections, and Miss Sladden's experiments on the heritability of induced feeding instincts in the stick insects have been published in the Proceedings of this Society. These are two of the most recent and best known of the experiments, but Harrison's experiments on forcing the saw-fly *Pontania* to adapt itself to laying its eggs on a new species and the heritability of this adaptation are equally convincing.

One of the arguments urged against Lamarckism is that it cannot account for what is called mimicry, that is the superficial resemblance of what is called a vulnerable species to one which is assumed to be protected by a bad taste, or odour, or an offensive weapon like a sting. This argument has been strongly stressed by Professor Hale Carpenter and it deserves respect, for it is hardly possible to conceive of this resemblance being due to a reaction on the part of the threatened animal. All one can say is that this subject deserves far more extensive study in the field than it has yet received. Eimer has investigated the gradual growth of the resemblance of the *Kallima* butterfly to a dead leaf and has shown that it is the outcome of a process common to a large number of butterflies and has nothing to do with this resemblance; this is the only adequate investigation of this subject known to me. It is quite uncertain in many cases whether the mimic really is protected by its appearance: this is

assumed not proved. If the theory of Natural Selection as applied to mimicry is true, then these mimicking species must produce random heritable variations in all directions, and, as we have seen, experimental evidence is dead against this assumption. But experiments have also shown that it is the pupal stage of insect life that is especially susceptible to the influence of the environment, and it should be possible to collect large numbers of the pupae of these mimics and rear them under the standard conditions to the imaginal stage, and it could then be seen whether these postulated deviations from the type in all directions really do occur, and whether they are heritable. The explanation of mimicry supported by Sir Edward Poulton and Professor Carpenter is really an ascription of the changes in the colour and shape of mimics to chance.

Dr. R. N. SALAMAN, F.R.S.—There is a wild tuber-bearing solanum, viz., *Solanum demissum*, which grows about 8000 feet above sea level in the Mexican mountains. It reproduces itself by seed, and morphologically the seedling plants are indistinguishable one from another. This plant through its seedlings has been under test at Ormskirk for some twenty years and the vast majority was found to be susceptible to wart disease (*Synchytrium endobioticum*), but generally there was a small proportion which was resistant. With the help of Mrs. McDermott of the National Institute of Agricultural Botany, a resistant strain has been selected and we are now in possession of an immune pure-breeding stock.

The point I wish to make is that this plant possessed a genetic recessive mechanism for resistance to wart disease, although it has lived for countless ages in an environment in which wart disease was entirely unknown. It cannot therefore be said that the environment had induced an adaptation; on the contrary, the basis on which adaptability to a new environment was artificially built up existed in the original stock.

This same plant exhibits another peculiarity: it is highly resistant to the fungus of common blight, *Phytophthora infestans*, a disease which, I understand, does not occur in these high altitudes of Mexico. This resistance has proved to be a dominant one, and we have now managed to transfer it by cross-breeding to the domestic potato.

Recently another form of the same plant, morphologically indistinguishable, which is susceptible to blight has been recovered from Mexico by Reddick.

Thus, in opposition to Professor McBride's views, we have in *S. demissum* a plant which is endowed with specific mechanisms of a Mendelian character against two distinct adverse agencies, neither of which is present in its native habitat.

Discussion on Theory of Natural Selection

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Nerve Excitation by Alternating Current

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I—INTRODUCTION

When an alternating current is passed through a nerve a relation exists between the intensity I necessary for steady threshold excitation, once to each cycle at the electrode considered, and the frequency n . For a pure sine-wave current applied to a nerve lying on two non-polarizable electrodes, and taking account of both time-constants (k of the local excitatory disturbance, λ of accommodation), this relation (Hill, 1936*a*, p. 343) should be:—

$$I/I_0 = \sqrt{(1 + 4\pi^2 k^2 n^2)(1 + 1/4\pi^2 \lambda^2 n^2)}, \quad (1)$$

where I_0 is the "true rheobase" for steady excitation by a regular series of constant-current pulses, each of sufficient duration to attain the minimum threshold.

The theory, in its present form, takes no account of the phenomena of absolute and relative refractoriness after an effective stimulus, so that equation (1) need not be expected to apply rigorously at frequencies so high that successive waves fall within the refractory periods, absolute and relative, of their predecessors. Nor can it allow for the fact that, owing to effects of electrical capacity at surfaces or membranes in the nerve, the current distribution in the nerve must change with change of frequency. It was important, however, to examine the relation between I and n over the wider range, and experiments were made at frequencies from nearly zero to 10,000 cycles per second. Those at the higher frequencies are referred to in § XI below; in the rest of the paper we deal only with the lower range (up to 300–1000 cycles per second, according to the temperature).

The theory takes no account of the "supernormal phase" of excitability (Adrian, 1920, 1921) which occurs under certain conditions, and may affect the threshold during repetitive stimulation of appropriate (rather low) frequency.

In fig. 1*a*, *b*, *c* the process of steady threshold excitation by sine-wave alternating current is illustrated graphically, for a typical case of frog's nerve at 15° C., and for three frequencies 10, 100, and 1000 cycles per second. The curves were drawn from equations (35) and (37) of a previous paper (Hill, 1936*a*). The full line represents the local potential (the local excitatory disturbance V), fluctuating with the applied current, but lagging behind it by a phase angle $\tan^{-1} 2\pi nk$ which is greater the greater the frequency. (A in each diagram denotes phase zero of the alternating current.) The broken line represents the threshold U , fluctuating with the applied current and with V , lagging behind the former by phase angle $(\tan^{-1} 2\pi nk + \tan^{-1} 2\pi n\lambda)$, behind the latter by phase angle $\tan^{-1} 2\pi n\lambda$.

Threshold excitation occurs when the V curve *touches* the U curve; if the former *crosses* the latter the stimulus is unnecessarily strong. The moment of such excitation is at E, the point where U is at phase zero; that is to say, at threshold excitation by alternating current the nerve is exactly unaccommodated (*see* Hill, 1936*a*, p. 342). For low frequencies the phase angle AE is small, for high frequencies it approximates to π ; for the optimum frequency it is $\pi/2$. Expressed otherwise, a threshold current of optimum frequency stimulates at the crest of each wave; of lower than optimum frequency, on the rising phase; of higher than optimum frequency, on the falling phase. These are deductions which could be tested experimentally. With an alternating current of any strength, both the local excitatory disturbance V and the threshold U

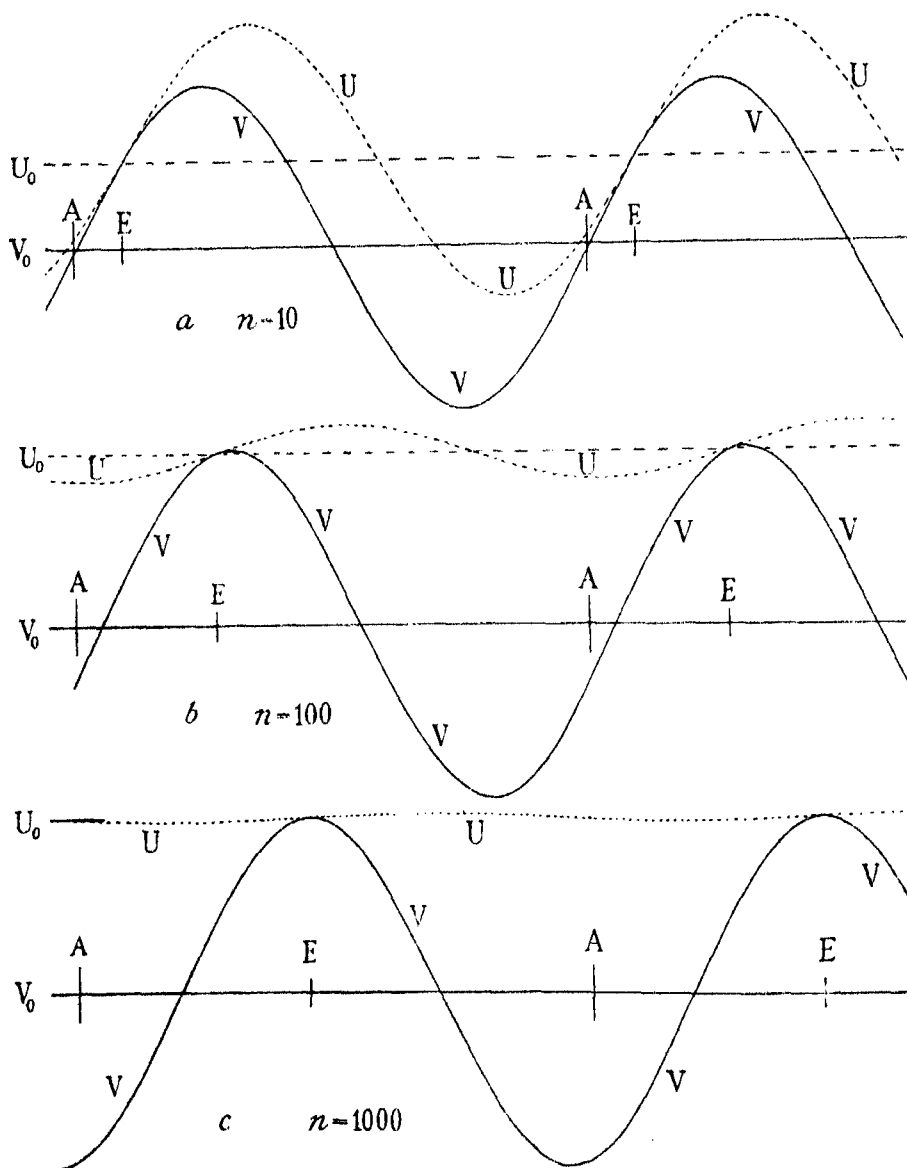


FIG. 1, *a*, *b*, and *c*—To show the time course of *V* and *U* in steady threshold excitation by alternating current, for $n = 10$, 100 and 1000 cycles per second respectively. *A* = commencement of A.C. wave; *E* = moment of excitation. Note (i) that *V* and *U* lag behind the current by a phase angle depending on the frequency, and (ii) that excitation occurs (at *E*) at the moment when $U = U_0$, i.e., when there is no "accommodation". The case taken (*cf.* Hill, 1936*a*, p. 342) is for a frog's nerve at 15° C. with $k = 0.5$ msec., $\lambda = 10$ msec. (fairly rapid accommodation). The currents required to excite in the three cases are, as multiples of the true rheobase: $n = 10$, $I/I_0 = 1.88$; $n = 100$, $I/I_0 = 1.06$; $n = 1000$, $I/I_0 = 3.32$. The optimum frequency is $n = 71$, for which $I/I_0 = 1.05$.

fluctuate more at a lower, less at a higher frequency. At a very high frequency, and with a current of threshold strength, V fluctuates with amplitude $(U_0 - V_0)$, while U scarcely fluctuates at all.

The optimum frequency n_{op} is given by

$$n_{op}^2 = 1/4\pi^2 k\lambda, \quad (2)$$

the strength I_{op} of the optimum stimulus by

$$I_{op}/I_0 = 1 + k/\lambda. \quad (3)$$

Equation (1) may be written,

$$I/I_0 = \sqrt{\left(1 + \frac{k}{\lambda} \frac{n^2}{n_{op}^2}\right) \left(1 + \frac{k}{\lambda} \frac{n_{op}^2}{n^2}\right)}, \quad (4)$$

or

$$I/I_{op} = \frac{\sqrt{\left(1 + \frac{k}{\lambda} \frac{n^2}{n_{op}^2}\right) \left(1 + \frac{k}{\lambda} \frac{n_{op}^2}{n^2}\right)}}{1 + k/\lambda}. \quad (4')$$

If we write $n/n_{op} = 10^x$, so that $x = \log_{10} n/n_{op}$, equation (4) becomes,

$$I/I_{op} = \frac{\sqrt{\left(1 + \frac{k}{\lambda} 10^{2x}\right) \left(1 + \frac{k}{\lambda} 10^{-2x}\right)}}{1 + k/\lambda}. \quad (4'')$$

The expression on the right of equation (4'') is unaltered by changing the sign of x . If, therefore, I be plotted against $\log_{10} n$ a symmetrical curve results, with its vertex at $I = I_{op}$ and its vertical axis of symmetry at $n = n_{op}$ (fig. 2). The symmetrical character of the $I - \log n$ curve was established experimentally by Coppée (1934c): it is confirmed by the experiments described in § IX below.

The relation between I/I_{op} and $\log n/n_{op}$ is invariable in all respects save one, viz., in the value of k/λ . If k/λ were constant, *i.e.*, if the behaviour of an excitable tissue were characterized by a single time-constant, then the relation in question would be completely invariable. The experiments given in § VIII show that it certainly is not; the reason is that k/λ , as pointed out by Solandt (1936a) on other evidence, can vary over a wide range. Coppée's experiments, indeed, already made this clear, for he refers to the fact (1934c, p. 16) that the *aplatissement* of his curves (the width of the $I - n$ relation at twice the minimum height) varied rather widely. In fig. 2 are three calculated curves of I/I_{op} versus $\log_{10} n/n_{op}$, for $k/\lambda = 1/10$, $1/30$, and $1/100$ respectively. Plotted on the same scales of I/I_{op} and $\log_{10} n/n_{op}$, the curves are more pointed the

greater the value of k/λ , more rounded the less that value. A 55% change in the width of the $I - \log n$ curve at twice the minimum height means, in this range, a 10-fold alteration of k/λ . In fig. 3a are observed points on a calculated curve for which $k/\lambda = 0.0624 = 1/16$ approximately.

The values of k and λ can be determined separately from the relation, experimentally observed, between I and n , provided that the said relation extends sufficiently far on either side of the optimum frequency. We

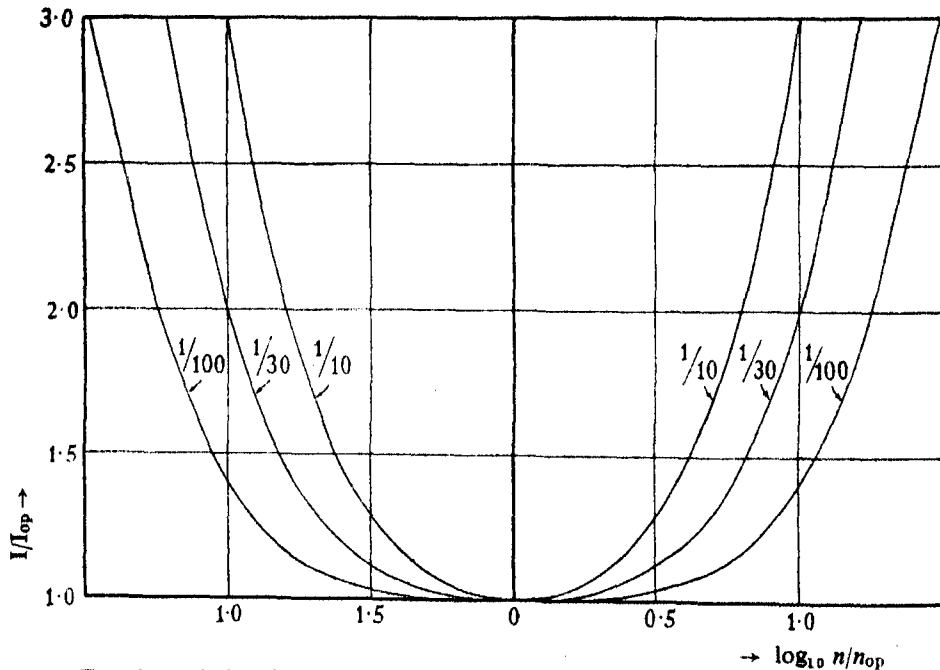


FIG. 2—Relation between threshold alternating current and the logarithm of the frequency, for different values of k/λ shown on the curves; drawn from equation (4'). Note that the vertices are made to coincide by dividing the current by the optimum current, and the frequency by the optimum frequency.

may, for example, plot I/I_{op} as observed against $\log n$ (fig. 3a) and then move about a family of curves, similar to that of fig. 2 but drawn on tracing cloth, over the observed points, until we get the best fit of the points with one of the family. The particular member of the family gives k/λ , while the optimum frequency gives $k\lambda$ (equation (2)): from these, k and λ can be calculated separately. This method works quite well, but a simpler one is as follows. This depends on the fact that k/λ is usually small, so that if either n^2/n_{op}^2 or n_{op}^2/n^2 is rather small, one or other of the factors on the right-hand side of equation (4) is nearly equal to unity. Thus if $k/\lambda = 1/20$ and $n^2/n_{op}^2 = \frac{1}{2}$ the effect on I^2/I_{op}^2 of putting

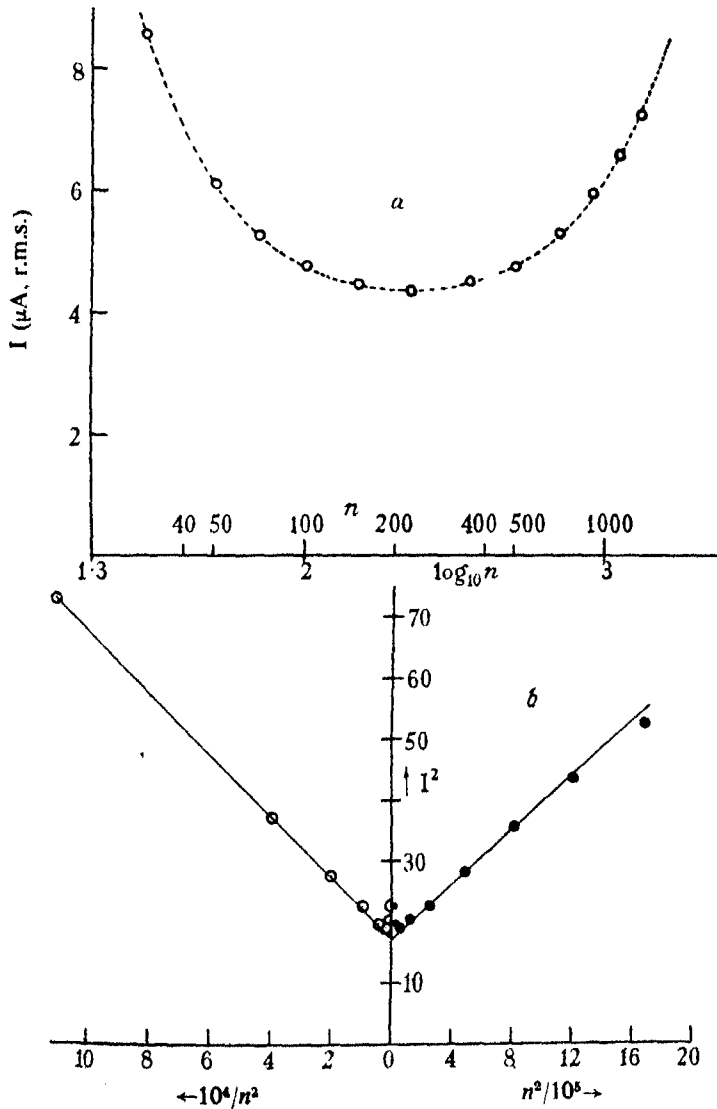


FIG. 3—Experiment of 9 January, 1936; Table XIV, § IX. Nerve treated with $6 \times \text{Ca}$, at 32°C . Muscle response as index. (a) I plotted against $\log_{10} n$, and a theoretical curve (from equation (1)) with the constants given in Table XIV. (b) I^2 plotted against (to the right) n^2 , and (to the left) $1/n^2$, showing linear relations on the high and the low sides respectively of the optimum frequency.

the first factor equal to unity is to make it only 2½% too small. Hence on the low side of the optimum frequency, with sufficient accuracy, equation (1) becomes,

$$I^2/I_0^2 = 1 + (1/n^2)/4\pi^2\lambda^2 \quad (5)$$

and on the high side,

$$I^2/I_0^2 = 1 + 4\pi^2k^2n^2. \quad (6)$$

Equations (5) and (6) mean that I^2 is a linear function of $1/n^2$ on the low side of the optimum, and a linear function of n^2 on the high side. The procedure, which is illustrated from an actual experiment in fig. 3*b*, is simply to plot I^2 against $1/n^2$ on the low side of the optimum, I^2 against n^2 on the high side; then to draw the best straight lines through the observed points. The intercepts on the I^2 axis give I_0^2 , the slopes of the two lines allow respectively λ and k to be calculated.

This method has been applied to a large number of experiments, and unexpectedly constant results have been obtained. The values of λ have been compared with those given by the standard method employing exponentially increasing currents; the values of k with those deduced from the standard strength-duration (voltage-capacity) relation. In both cases the agreement is good.

The optimum frequency being given by the relation $n_{op}^2 = 1/4\pi^2k\lambda$, is low if $k\lambda$ is large. The usual reason for $k\lambda$ being large is that λ is large. With a large value of λ a long-lasting current is apt to evoke repetitive response (Katz, 1936*b*, *c*), so that at very low frequencies each wave produces a whole series of impulses in the nerve; this may make it impossible to obtain reliable results on the low side of the optimum. There is another objection, a technical one, to working with very low frequencies, viz., that even the best valve-oscillator will not give a pure enough sine-wave current at such frequencies. Other apparatus may be employed in this region, as Coppée (1934*c*) did, and as we have done, but an oscillator is by far the most convenient; for both reasons, therefore, most of the evidence given below was obtained with an oscillator, at frequencies above 25 cycles per second, and with nerves used under such conditions as to have optimum frequencies of 60 cycles per second and over.

II—EXPERIMENTAL

Preparation—The sciatic nerves of large Hungarian frogs (*Rana esculenta*) were employed throughout. Those of English frogs were not long enough for convenient use and for the avoidance of leaks when the action current was taken as index of response. The work has extended over 16 months, so that animals at various seasons have been used. The

latest and most decisive experiments were made during the winter and spring (January–April) of 1936 on a batch of frogs which arrived in the laboratory in October, 1935, and were kept in the open air in a pond on the roof. They were usually placed in a sink indoors for a few days before use. In some of these experiments frogs were employed which, recently caught, arrived from Hungary in March. The experiments of December, 1934, to June, 1935, were on frogs which had been in the laboratory since October, 1934, some in an aquarium, some (for the later experiments) in a cold store at about 5° C.; the latter were kept in the warm for a few days before use. No consistent differences were noted.

In many experiments the nerve only was used, its electric response being taken as index of excitation. In others, a muscle-nerve preparation was employed, muscle response to nerve excitation being observed. The preparation, in either case, was soaked before use in Ringer's fluid, "normal Ringer" consisting of 6.75 gm. NaCl, 0.2 gm. CaCl₂, 0.15 gm. KCl, made to 1 litre with distilled water. In many experiments, as noted below, CaCl₂ was added to the Ringer's fluid: 10 × Ca (*e.g.*) denoted that the Ringer's fluid contained 10 times the normal concentration of Ca. For a few experiments, a Ringer's fluid poor in Ca (*e.g.*, 0.4 × Ca) was made up; for others, the animal had 40 to 100 mg. of CaCl₂ in isotonic solution, injected into its dorsal lymph sac on the previous day. The nerves from Ca-injected frogs were soaked in a high-Ca Ringer's solution before use.

Electrodes—Calomel half-cells were employed throughout, both for stimulating and for recording the action current. A galvanometer placed in the stimulating circuit gave no sign of rectification of the alternating current. The calomel half-cells dipped into pools of Ringer's fluid. Contact was made with the nerve by sharp-edged strips of soft wood, soaked for a long time previously in normal Ringer's fluid. When the nerve was soaked in abnormal Ringer's solution the soft wood strips were soaked in it too, and the pools were filled with it. The strips were held fast by being pushed into plasticine at the bottoms of the pools. Their edges were usually about 20 mm. apart, but in certain experiments (§ V) they were brought as close as 1.5 mm. (*see* Hill, 1936*b*, pp. 443, 448; Solandt, 1936*a*, p. 357).

Chamber—This was fashioned from a block of paraffin wax. For a muscle-nerve preparation there were two (or three) pools, dug out in the wax, for the calomel half-cells. The muscle was pinned to a cork sunk in the wax. A glass cover, preferably with a strip of moist filter paper on

it, was used to prevent evaporation. The muscle was connected to a spring lever, for ease in observing the response. For a nerve preparation, there were four (or five) pools for the calomel half-cells, two being used for leading off the action current.

Galvanometer—A very sensitive moving-coil galvanometer, of period about 4 seconds, was used for recording the monophasic action current. This was critically damped with an external resistance of about 2000 ohms. Maximal stimulation at moderate frequency gave a steady deflexion of 50 to 200 mm. As index of constant response, either a small galvanometer deflexion (1 to 3 mm.) or (at first) a constant small fraction of the maximal response for the frequency considered was taken. The latter was later discarded, since at high frequencies the stimulus for maximal response is so great that it tends to alter the threshold, and at very high frequencies the true maximal response cannot be determined (*see* Katz, 1936a).

Oscillator—For the earliest experiments an oscillator constructed by the Cambridge Instrument Company was used. This was calibrated from 50 to 3000 cycles per second with the aid of tuning forks. It was tested with a cathode ray oscillograph (by the kindness of Professor D. T. Harris) and the conditions necessary in order to obtain an approximately pure wave-form were worked out. At low frequencies, however, the wave was never sufficiently pure, and the range was not wide enough. With the aid, therefore, of a grant from the Royal Society, a Beat-Tone Oscillator of N.P.L. design (Type 3A) constructed by Messrs. Muirhead & Co., of Beckenham, Kent, was obtained. This was calibrated by the National Physical Laboratory for frequency, from 10 to 10,000 cycles per second: it can be set precisely from time to time by a "trimming condenser" and two reeds contained in the equipment. The root mean square value of all the upper harmonics in the wave given by a Muirhead amplifier connected to the oscillator, according to the N.P.L. certificate, is given in Table I. For the present investigation the oscillator was used without the amplifier, so that the harmonic content was presumably less. The oscillator has six ranges, controlled by a potentiometer in the input circuit of the detector valve, and to keep the wave as pure as possible the highest range (No. 6), which gives a rather large grid swing to the last valve, was not used, except sometimes at high frequencies when the whole available E.M.F. was required.

The oscillator was carefully tested at low frequencies (10 to 200 cycles per second) with a Downing moving-coil oscillograph viewed with a set

of revolving mirrors. Below 20 cycles per second the wave form was perceptibly distorted, but at and above 25 cycles per second a very good wave was obtained. It is essential for the argument that a pure sine-wave should be used, otherwise (particularly at low frequencies) entirely false thresholds may be obtained. The wave-form, for example, of Coppée's earlier oscillator (1934a, fig. 5, p. 245) might well lead to anomalous results.

TABLE I—HARMONIC CONTENT OF WAVE, AS A PERCENTAGE OF THE FUNDAMENTAL

	Cycles per second	%
Range 4	100	3.0
	500	2.5
	2000	<1
Range 5	100	4.5
	500	4.1
	2000	2.2
Range 6	100	6.1
	500	5.7
	2000	3.1

For very low frequencies an apparatus was constructed by which the total light through an aperture was made to vary sinusoidally with the time. The frequency could be varied from zero upwards to about 250 cycles per second. The light was concentrated by a condensing lens into a vacuum photo-cell, which was connected to an amplifier. The form of the wave so produced was tested while running by photographing it with an oscillograph, or at "zero" frequency by setting the disk (which was calibrated round its edge), in a series of positions, reading the current with a galvanometer, and plotting. The wave form was good and was independent of frequency. The apparatus worked well but was much slower to set and less convenient to use than the oscillator, since variation of frequency meant stopping a motor and altering belts on pulleys.

The photoelectric arrangement need not be further described here, since the results obtained with it are the less important, and analogous systems have been described before (*see e.g.*, Nicolai, 1930, and the mechanical arrangements of von Kries, 1884a, and of Coppée, 1934c).

The Measurement of the Current—In the later experiments a Westinghouse "instrument type" rectifier connected to a microammeter, together with a potentiometer arrangement of Muirhead reactionless resistance

boxes, was employed (fig. 4). The rectifier was calibrated at various frequencies, up to the highest used, and with various intensities of current, against a pair of Cambridge "vacuo-junctions" (in series) connected to a very sensitive moving-coil galvanometer. The vacuo-junctions themselves were calibrated with direct current measured by the same microammeter. It was found that the "1 mA." rectifier could be used safely to well below 100 μ A., and at all frequencies from 25 to 10,000 cycles per second.

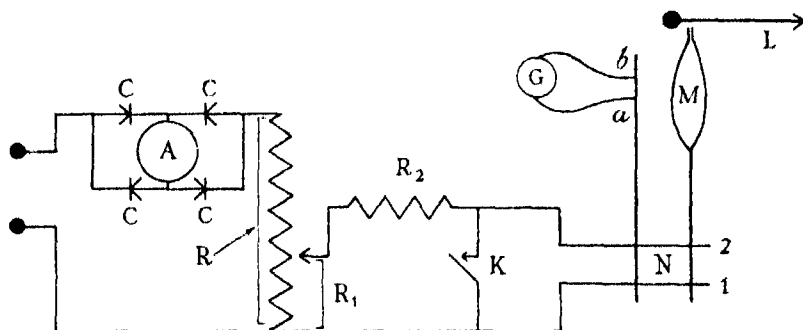


FIG. 4—Diagram of connexions for adjustment, and measurement of strength, of alternating current stimulus. *R*, reactionless resistance box, 110,000 ohms. *R*₁, adjustable part of *R*. *R*₂, calibrated "grid leak" resistance, about 1 megohm. *C*, Westinghouse 1 mA. metal rectifier, instrument type. *A*, microammeter connected to rectifier. *G*, sensitive galvanometer for recording monophasic action-current. *K*, short-circuit key. *L*, lever to muscle *M*. *N*, nerve on stimulating non-polarizable electrodes (1) and (2), (1) being in a well-injured region so that excitation occurred only at (2); connected either to muscle or galvanometer. *a*, *b*, non-polarizable electrodes, the nerve being well injured at *b* for monophasic response. Note.—*R*₂ was so large that opening *K* did not affect the current read on the microammeter.

The calibration number to turn "average" rectified current, measured on the microammeter, into root-mean-square is theoretically $\pi/2\sqrt{2} = 1.11$, (i) if the wave-form is perfect, (ii) if there is no frequency error due to capacity in the rectifier, and (iii) if there is no appreciable leak in the rectifier with the strength of current used. Actually several calibrations carried out at intervals during the course of the work gave extreme values of 1.11 and 1.14, with a mean of 1.13 which has been used throughout. The consistency of the calibrations is a tribute to the rectifier, and (if one were needed) to the purity of the wave-form. See Table II.

In the comparison of alternating current with other forms of stimulation it is the "crest" value *I* which is needed, for the actual current at time *t* has been written *I* sin 2 π *nt*. With a pure sine-wave current the

crest value is $\sqrt{2}$ times the r.m.s. value, so that the factor to turn "average" rectified current into "crest" current was $1.13 \times \sqrt{2} = 1.60$.

The current i from the oscillator, read by the rectifier and microammeter, was usually about $100 \mu\text{A}$. Let R_1 be the resistance, from the ends of which current is led off to the nerve. Let R_n be the resistance of the nerve with electrodes; R_2 the resistance of the "grid-leak" in series with it. Then the current I through the nerve is given by

$$I = R_1 i / (R_1 + R_2 + R_n).$$

TABLE II—CALIBRATION OF WESTINGHOUSE RECTIFIER ("1 mA., INSTRUMENT TYPE") WITH TWO CAMBRIDGE VACUO-JUNCTIONS CONNECTED TO VERY SENSITIVE MOVING-COIL GALVANOMETER. THE FACTOR IS THAT REQUIRED TO TURN RECTIFIED CURRENT INTO ROOT-MEAN-SQUARE.

Cycles per second	50	150	500	1500	4000	10000
Current, μA ., (r.m.s.) . .	89	93	95	92	78	44
Factor	1.13	1.135	1.13	1.135	1.135	1.135

The resistance of the nerve and electrodes was measured at the end of an experiment. It was usually about 60,000 ohms, a comparatively small fraction of the resistance in series with it. The resistance (strictly, the impedance) of the nerve varies somewhat with frequency (*see below*), but this variation is altogether swamped by the high resistance R_2 in series with it.

Since R_1 could be as high as 110,000 ohms and R_2 was about a megohm, and since i could be as great as $200 \mu\text{A}$. (r.m.s.), the maximum current available through the nerve was about $20 \mu\text{A}$. (r.m.s.). This is about 20 times the average threshold for a normal nerve at optimum frequency, 7 times that for a nerve treated with Ca: it allowed us usually to go to as high a frequency as was needed; when necessary the resistance R_2 was reduced to 500,000 ohms.

The resistance R (Muirhead's "Universal" resistance boxes with non-reactive windings) should show no measurable effects of capacity or inductance up to the highest frequency we have used. According to the makers, indeed, the percentage error in impedance is completely negligible up to 50,000 cycles per second. The leads to the rectifier and to the nerve were kept well apart to avoid effects of capacity. The effects of inductance are negligible in circuits of such high resistance.

In the earlier experiments a Cambridge vacuo-junction connected to a sensitive galvanometer was used instead of the rectifier, the rest of the

connexions being analogous to those in fig. 4. This was accurate but not nearly so quick or convenient.

It would have been simpler, had it been possible, to avoid the potential division of fig. 4, to vary the current simply by varying a large series resistance, and to put the rectifier directly in the nerve circuit. This could not be done, since rectification is not complete with currents as low as the $1 \mu\text{A}$. required for the minimum threshold of isolated nerve.

Leaks and Earth Connexions—The precautions to be taken under this heading are among the most important of all for alternating current stimulation, but there is little mention of them in the literature of the subject. Where our results differ from those of others we are inclined to attribute the divergence, in some cases at least, to neglect of necessary precautions. A capacity of various parts of one's apparatus to earth can provide another path for an alternating current, particularly of high frequency, and when one is dealing with an indicator as sensitive as an isolated frog's nerve a fraction of a microampere may altogether spoil one's results.

The same effect might occur with very rapid condenser discharges; expressed mathematically, the Fourier analysis of a series of condenser discharges would involve very high harmonics, and these, if there were earth capacities to various parts of one's apparatus, might—by leaking across via the earth—cause considerable disturbances. The constant-quantity relation for very short times has probably been long obscured for such reasons; even the simplicity of the strength-duration (voltage-capacity) relation in frog's medullated nerve has probably escaped detection by the same cause.

It is not possible to avoid leaks of this kind by interposing even the best condensers in the two lines from one's oscillator; these can eliminate only constant current disturbances. The ordinary laboratory bench, although apparently dry, is often a serious cause of trouble; it may act as a large earthed plate of a condenser, of which the other plate is some part of one's apparatus. Trouble from this source can be avoided by heating the laboratory bench and tables (*e.g.*, by radiators placed beneath them) and then a suitable earth connexion to some part of the stimulating circuit, found by trial, usually eliminates any further disturbance. Large earthed objects near any part of the stimulating circuit are to be avoided, and any sign of leaks to the nerve with high frequency current should be explored and the experiment proper be postponed until the leaks have been "earthed". Batteries, not D.C. power mains, should be used for the oscillator, for the mains invariably cause trouble by leaks. These and other elementary precautions were taken in all the later experiments, and the consistency of the results is partly due to them.

Nerve Impedance and Frequency—In discussing the stimulating circuit shown in fig. 4 we referred to the well-known fact (*see, e.g., Lullies (1930a)*) that the impedance of nerve varies with frequency, and therefore that the series resistance (R_s) must be high compared with that (R_n) of the nerve. A few determinations were made of the variation of impedance with frequency, under the conditions of the present experiments. A substitution method was used. Current from the oscillator, measured by the rectifier connected to a quick mirror-galvanometer, was passed either through the nerve or through a reactionless resistance box, and the resistance was varied until the same reading was given by either. From the resistance so determined was subtracted that of the electrodes alone, when connected together either by a thick piece of filter paper wet with Ringer's fluid, or by direct contact at their tips; the electrode resistance was independent of frequency. In one typical experiment at 30° C. the following results were obtained, for 15 mm. and 1.5 mm. respectively of nerve.

n	30	500	2000	10000
R_n (15 mm.)	30250	28500	26700	24800
R_n (1.5 mm.)	9000	8900	8200	7200

In another at 29° C., for 22 mm. and 2 mm. respectively:—

n	30	100	500	1000	2000	5000	10000
R_n (22 mm.)....	67300	67000	66000	64700	63000	60800	59500
R_n (2 mm.)	15000	14700	14300	14000	13300	12500	11800

In another at 27° C., for about 6 mm. length:—

n	20	50	100	200	500	1000	2000	5000	10000
R_n	42200	42000	41800	41900	41700	40900	39700	38200	37100

In eleven such determinations the impedance fell, on the average, about 18% between 30 and 10,000 cycles per second. With a nerve of 50,000 ohms and a total resistance of rather over a megohm the variation of nerve impedance with frequency, between the extreme limits of frequency used, is less than 1% of the total impedance, and so can be neglected. If, however, the resistance R_s were small, the variation with frequency would not be negligible, and serious error might result.

III—THE COMPARISON OF THE OPTIMUM CURRENT WITH THE RHEOBASE, AND OF THE "TRUE" WITH THE "OBSERVED" RHEOBASE

According to the theory (Hill, 1936a) the threshold current I_{op} at the optimum frequency n_{op} is related to the "true rheobase" I_0 by the

equation $I_{op} = I_0 (1 + k/\lambda)$. If, therefore, I_{op} and k/λ be measured, I_0 can be calculated. Moreover, as shown above, I_0 can be obtained by plotting I^2 against either (i) $1/n^2$ on the low side of the optimum, or (ii) n^2 on the high side of the optimum, when I_0^2 is the intercept of either of the straight lines so obtained on the vertical axis. It is interesting to compare the values of I_0 determined by these methods with I_1 the "observed rheobase".

In measuring the rheobase I_1 it is not sufficient to find the threshold for a single response to a single constant current, since the threshold may be appreciably, sometimes considerably, lower for repetitive stimulation than for a single stimulus. The reason for this is not known, but the fact itself is certain as the following experiments show. A frog's muscle-nerve preparation was placed in the usual chamber at 18.5 to 19.5° C., with the nerve on non-polarizable electrodes. The rheobase for a constant current was determined in the ordinary way, the index being a single minimal twitch. A series of constant current pulses was then sent into the nerve, at a frequency of 40-22 per second, each pulse being of duration $2\frac{1}{2}$ - $4\frac{1}{2}$ msec., which was definitely greater than the *Hauptnutzzeit*, so that the minimum threshold was attained. With a rather large value of λ , the interval, viz., 22-41 msec., between the pulses might not be quite long enough for the accommodation of the nerve to be fully reversed; in some of the experiments, therefore, in order to avoid any accumulating accommodation to repetitive one-way pulses, or any possible polarization in the nerve itself, each pulse was followed, after an interval of 0.5-1.0 msec., by a reverse pulse of precisely the same strength and duration in the opposite direction. The cathode of the reverse pulse was in an injured region of the nerve so that its threshold was much higher; thus no excitation could result from the reverse pulse, which was used only to wipe out any possible after-effect of its predecessor and to leave the nerve more or less in its resting state. The cycle was therefore as follows:—

Pulse $2\frac{1}{2}$ - $4\frac{1}{2}$ msec.; interval 0.5-1.0 msec.

Reverse pulse $2\frac{1}{2}$ - $4\frac{1}{2}$ msec.; interval 20-35 msec.

Excitation at break did not occur at the end of the reverse pulse, as shown by the fact that a series of reverse pulses only had no effect except with a much stronger current.

The index taken in the case of the repetitive pulses was a minimal steady tetanus. The threshold strength I_1 was measured and compared with that (I_1') for a single constant current. I_1 was always less than I_1' , sometimes considerably less, as the results in Table III show. There is clearly

some accumulating, or "facilitating", effect of a series of subthreshold pulses, which finally become adequate at a lower threshold than a constant current. This effect has no explanation in the present theory of excitation, but it is evident that it must be taken into account when comparing the threshold for alternating current with that for constant current. Alternating current, as used, provides one stimulus to each cycle, and in any comparison with another form of current the latter should, if possible, be employed in a way which also permits repetitive stimulation.

The experiments in Table IV were performed in January, 1936, on the relation between the optimum threshold I_{op} by alternating current and the rheobase I_1 by repetitive pulses. k and λ were separately determined by equations (5) and (6), and so the "true rheobase" I_0 was calculated

TABLE III—COMPARISON OF RHEOBASE FOR CONSTANT CURRENT (I'_1) WITH RHEOBASE FOR REPETITIVE PULSES (I_1)

	*	*	*	*		
Preparation number	1a	1b	2a	2b	3a	3b
I_1/I'_1	0.95	0.94	0.92	0.92	0.96	0.95
			*			
Preparation number	4a	4b	5a	5b	6a	6b
I_1/I'_1	0.92	0.93	0.76	0.87	0.92	0.84

1 megohm in series with nerve.

* Two-way pulses to avoid accommodation or polarization—see text.

from $I_0 = I_{op}/(1 + k/\lambda)$. In all that follows each of the repetitive pulses was followed by a reverse pulse, to avoid polarization or accommodation, as described above.

The mean values, for the 16 experiments of Table IV, are:—

of I_{op}/I_1	1.05
of I_0/I_1	0.985

The experiments of Table V were performed in January and February, 1936, on the ratio I_0/I_1 , where I_0 is the "true rheobase" obtained by alternating current as described above (equation (5)) by extrapolation on the low side of the optimum frequency, and I_1 is the rheobase observed by pulses.

The mean value of I_0/I_1 for the 15 experiments of Table V is 1.02.

The experiments of Table VI were performed in January and February, 1936, on the ratio I_0/I_1 , I_0 in this case being obtained (equation (6)) by extrapolation on the high side of the optimum frequency. The mean value of I_0/I_1 for the 19 experiments of Table VI is 0.995.

TABLE IV—COMPARISON OF OPTIMUM STIMULUS WITH RHEOBASE

	31*	29*	16	29	28	28	28	27	27
Temperature, °C.									
I_{op} , $\mu A.$, crest	4.13	2.98	1.87	2.47	1.78	1.86	1.81	1.81	1.30
n_{op} , cycles per sec.	200	190	54	155	140	150	200	188	188
I_1 , pulses, $\mu A.$	3.76	2.75	1.62	2.10	1.63	1.65	1.815	1.31	1.31
Pulse frequency, per sec.	80	80	78	80	57	57	61	60	60
I_{op}/I_1	1.10	1.08	1.15	1.18	1.09	1.13	1.00	0.99	0.99
k/λ	0.107	0.10	0.037	0.069	0.07	0.12	0.078	0.061	0.061
I_0 , $\mu A.$, crest	3.73	2.71	1.80	2.31	1.67	1.66	1.68	1.23	1.23
I_0/I_1	0.99	0.99	1.11	1.10	1.02	1.01	0.84	0.925	0.93
Temperature, °C.	29	31*	24	21	23*	22	23*	Mean	
I_{op} , $\mu A.$, crest	2.35	6.2	1.34	1.55	7.14	1.36	5.05	—	—
n_{op} , cycles per sec.	186	245	128	40	95	77	102	—	—
I_1 , pulses, $\mu A.$	2.31	5.9	1.33	1.57	6.55	1.40	4.80	—	—
Pulse frequency, per sec.	60	60	44	42	43	43	43	—	—
I_{op}/I_1	1.02	1.05	1.01	0.99	1.09	0.97	1.05	1.05	1.05
k/λ	0.085	0.051	0.074	—	0.03	0.069	0.032	—	—
I_0 , $\mu A.$, crest	2.17	5.90	1.25	1.53	6.93	1.27	4.90	—	—
I_0/I_1	0.94	1.00	0.94	0.97	1.06	0.91	1.02	0.985	0.985

* Signifies a nerve treated with high-Ca concentration.

TABLE V—COMPARISON OF "TRUE" WITH "OBSERVED" RHEOBASE FROM LOW SIDE OF OPTIMUM

Temperature, °C.	34	24	24	23	23	24	24	24	25	24	24	25	24	25	26	27
I_0/I_1	1.04	0.99	0.99	1.06	1.02	1.06	1.05	1.02	1.03	1.00	1.02	1.00	1.03	0.96	1.02	1.02

In most of these experiments the pulses were 43 per second and of duration 2.6 msec.

TABLE VI—COMPARISON OF "TRUE" WITH "OBSERVED" RHEOBASE FROM HIGH SIDE OF OPTIMUM

Temperature, °C.	30	34	34	32	32	32	23	23	24	24	23
I_0/I_1	0.86	1.04	1.01	1.04	1.02	0.99	0.90	0.95	0.99	1.07	1.07
Temperature, °C.	22	23	24	24	24	24	25	25	26	26	26
I_0/I_1	0.92	1.02	1.00	1.02	1.03	1.00	1.01	1.00	1.00	1.00	1.00

The net result of these 50 determinations by three different methods is that the mean value of I_0/I_1 is exactly unity, 35 of them (70%) being between 0.95 and 1.05. The mean value of I_{op}/I_1 , in 16 experiments, was 1.05. This is in complete disagreement with Coppée's conclusion (1934c, p. 14) that "the threshold crest potential at the optimum frequency is always less than the rheobase", and his statement that in most of his experiments the former was 65% to 85% of the latter. The divergence must be due in part to the fact that Coppée neglected to use repetitive pulses of constant current to determine the rheobase, so that his observed rheobase was too high (see Table III above) and his ratio too low.

In § IV it is shown that the "true rheobase" I_0 deduced from alternating current measurements is approximately equal to the rheobase extrapolated from the E-RC relation with repetitive condenser discharges, being probably slightly less. The results were rather more variable than those just referred to, but they add general confirmation to them.

The fact that the mean value of I_0/I_1 is unity was not expected. According to the theory (Hill, 1936a, pp. 321, 322, 325) I_0/I_1 is less than unity in the ratio $1: (\lambda/k)^{\frac{1}{\lambda/k-1}}$; for a usual value of λ/k of (say) 25, I_0/I_1 should be 0.88, for $\lambda/k = 100$ I_0/I_1 should be 0.95, for $\lambda/k = 10$ I_0/I_1 should be 0.78. This quantitative divergence from the theory is probably related to another observed fact, viz., that the "*Hauptnutzzeit*" is nearly always rather greater than predicted (Hill, 1936a, p. 322). For $\lambda/k = 40$, the calculated utilization time of a threshold constant current is $3.79k$, which for $k = 0.35$ msec. is 1.33 msec. A more usual experimental value is about 2 msec. Dr. H. Rosenberg informs us that in experiments which he made in April, 1929, on frogs' nerves at about 18° C., the make-response times ranged from 1.31 to 1.98 msec., for currents which gave just noticeable action-potentials. The low value occurred only once, and it is probable that the current was slightly above the threshold and that the longer durations are more correct. Thus the theory gives rather too high an "observed" rheobase I_1 and rather too short a "*Hauptnutzzeit*".

It was emphasized (Hill, 1936a, pp. 319, 346) that the theory takes no account of electrotonic changes of excitability (Pflügers law) and that in consequence exact quantitative agreement with experiment may not always be found. The two examples just mentioned are probably of this kind. The process of accommodation tends to make the threshold higher, the electrotonic increase of excitability at the cathode tends to make it lower, and apparently for currents of rheobasic strength *on the average* the two effects just balance one another at the utilization time. Fig. 5 describes diagrammatically the supposed interaction of accommodation and the electrotonic rise of excitability. The full line

shows U and V (threshold and local potential) according to the original theory (cf. Hill, 1936a, fig. 3). The broken lines illustrate how a fall of threshold due to electrotonus, and setting in rapidly, followed by the usual rise due to accommodation, may (i) lower the observed rheobase until it is approximately equal to the true rheobase, and (ii) increase the utilization time of a current of rheobasic strength.

There would be no value at present in a mathematical discussion of the effect of electrotonus in this connexion, since not enough is known

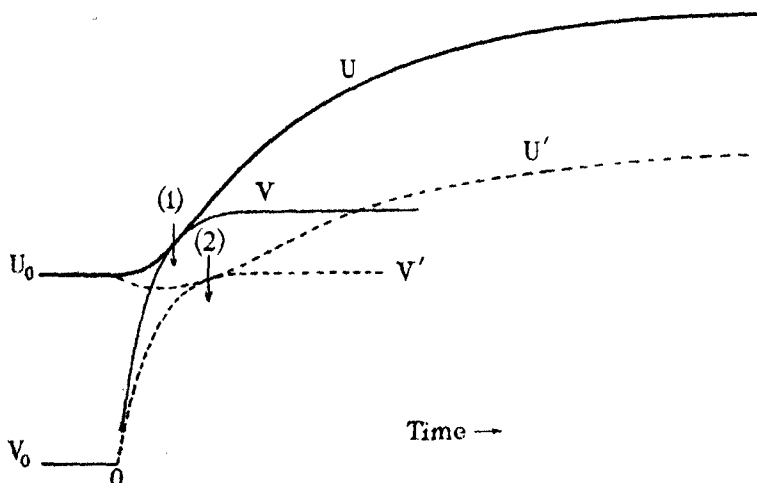


FIG. 5—Diagram to illustrate the effect of electrotonic increase of excitability on "utilization time" of threshold constant current. Changes of local potential (V) and of threshold (U) as functions of time on making the current at O . Full lines show "normal" accommodation, in which the final value of ($U - V$) is the same as the initial value ($U_0 - V_0$); arrow (1) at moment of excitation. Broken lines show the usual effect of electrotonus, in which the final value of ($U' - V'$) is less than the initial value ($U_0 - V_0$); arrow (2) at moment of excitation. The initial downward trend of U' is supposed to be due to electrotonus setting in early and lowering the threshold quicker at first than accommodation raises it. The effect of this is to increase the "*Hauptnutzzeit*".

about it. It is clear, however, that it works in the required direction and is of the right order of size to explain the observed effects.

IV—THE COMPARISON OF k CALCULATED FROM THE INTENSITY-FREQUENCY RELATION WITH ALTERNATING CURRENT, WITH k CALCULATED FROM THE STRENGTH-DURATION (VOLTAGE-CAPACITY) CURVE

A crucial test of the theory from which equations (1) and (6) were deduced was to compare the value of k calculated by equation (6) from

experimental data with alternating current, with that deduced from an observed strength-duration (voltage-capacity) relation. In the former, I^2 was plotted against n^2 on the high side of the optimum frequency, and k and I_0 were calculated from the linear relation obtained. (The procedure is illustrated in fig. 3*b* and fig. 9*b*.) In the latter, $\log_{10} E$ was plotted against $\log_{10} RC$ and the best fit of the theoretical relation obtained by the procedure described elsewhere (Hill, 1936*b*, p. 441). This gave k and E_0 . In both cases a minimal steady muscular contraction was used as index of constant response of the nerve, the condenser discharges being one-way at about 50 per second.

A very complete system of "reverses" eliminated the effects of any progressive change in the preparation during an experiment. Most experiments began and finished with alternating current. First a series of readings was made at various selected frequencies, increasing from about the optimum upwards; this series was then repeated in the reverse order at the same frequencies. Next a series of about nine readings was made with condensers, for the E - RC relation, usually from $RC = 10$ msec. to $RC = 20$ μ sec. This series also was then repeated in the reverse order. Finally a second complete series of readings (with "reverse") was made with alternating current.

At every frequency n , therefore, of the alternating current there were four readings of the intensity I , suitably spaced in time to ensure the elimination of any effect of progressive change in the preparation. The mean value of I at each n was used in the calculation. Similarly for each RC with the condenser discharges there were two values of E , of which also the mean was taken. The consistent results obtained were doubtless due, in part, to this systematic elimination of the effect of any gradual alteration in the preparation during an experiment.

No attempt was made to determine k by a single pair of readings, as in "chronaxie" measurements; a whole curve was always recorded.

The main series of experiments was made during February and March, 1936, at various temperatures from 16.5° to 27° C. In every experiment, at least a reasonably good linear relation between I^2 and n^2 was obtained with alternating current, permitting no doubt of the value of k : similarly the E - RC results fitted the theoretical curve with the same order of accuracy as was described elsewhere (Hill, 1936*b*).

Table VII gives a summary of these results. The mean of 18 values of k from alternating current is 0.44 msec.: that of the corresponding values of k by condenser discharges is 0.48 msec. In some experiments the difference is well beyond the limits of experimental error; we suggest that in these, for some unknown reason, the alternating current and the

TABLE VII—COMPARISON OF k (AND I_0) BY ALTERNATING CURRENT AND BY CONDENSER DISCHARGES

Temperature °C.	k (A.C.) msec.	k (condensers) msec.	I_0 (A.C.) μ A.	I'_0 (condensers) μ A.	I_0/I'_0	Remarks
16.5	0.46	0.52	1.21	1.28	0.94	—
18	0.51	0.56	1.30	1.31	0.99	—
20	0.47	0.48	1.41	1.64	0.86	—
19	0.49	0.53	1.37	1.44	0.95	—
19	0.42	0.33	2.48	2.82	0.88	8 $\times$ Ca
19	0.28	0.38	2.53	2.33	1.08	8 $\times$ Ca
20	0.67	0.69	0.82	0.93	0.88	—
20	0.57	0.62	1.11	1.04	1.07	—
22	0.31	0.45	3.77	3.26	1.16	8 $\times$ Ca
21	0.46	0.50	2.73	3.12	0.87	8 $\times$ Ca
21	0.57	0.48	—	—	—	—
23	0.13	0.16	2.93	3.86	0.76	8 $\times$ Ca
21	0.58	0.51	1.35	1.83	0.74	8 $\times$ Ca
22	0.52	0.71	0.89	0.85	1.05	—
22.5	0.32	0.32	3.54	3.26	1.09	8 $\times$ Ca
23	0.55	0.52	0.99	1.42	0.70	—
25	0.35	0.50	1.64	1.79	0.92	—
27	0.275	0.385	1.80	1.72	1.05	—
Mean 21° C.	0.44	0.48	—	—	0.94	—

condenser discharges were exciting different fibres in the nerve. On the average, however, the agreement is so close as to leave no reasonable doubt that the same quantity is being measured by both methods. The ratio of I_0 (crest) deduced from the alternating current readings to I_0' deduced from condenser readings has a mean value of 0.94, but the individual readings are rather variable and the standard deviation of the mean is ± 0.03 . The approximate equality of I_0 and I_0' was referred to in § III above.

The series of experiments in Table VII was made under conditions carefully standardized as the result of long experience; a number, however, of earlier experiments had given the same result, and since these were made under a variety of conditions their evidence adds weight to the conclusion from the others. For example, in December–January, 1934–35, using the Cambridge oscillator and the electric response of the nerve as index, the results given in Table VIII were obtained.

TABLE VIII

Temperature ° C.	Room	20	21	19	6
k (A.C.) msec.....	0.30	0.58	0.38	0.51	0.93
k (condensers) msec.	0.35	0.54	0.32	0.59	0.89

Later, in May and June, 1935, with the Muirhead oscillator and the electric response of the nerve as index, the results given in Table IX were obtained.

TABLE IX

Temperature ° C.	6	19	6
k (A.C.) msec.....	0.80	0.40	0.72
k (condensers) msec.	0.92	0.38	0.62

The mean ratio of k (by A.C.) to k (by condensers) in these eight experiments is 1.01, which confirms the conclusion from the experiments in Table VII that there is, on the average, no significant difference between the k 's measured by the two methods.

According to Coppée (1934*b*; 1934*c*, p. 20) the chronaxie is equal to one-third of the period of the "isopotential frequency", the latter being defined as the frequency (above the optimum) for which the threshold (root-mean-square) is equal to the constant current rheobase. The crest value of such a current is $\sqrt{2}$ times the rheobase. We have found above (§ III) that, for repetitive constant current pulses, the observed rheobase I_1 is equal to the true rheobase I_0 . Hence if I is the "isopotential current", from equation (6) (the frequency being well above the optimum),

$$I^2/I_1^2 = I^2/I_0^2 = 2 = 1 + 4\pi^2 k^2 n^2.$$

Therefore $4\pi^2 k^2 n^2 = 1$ and $k = 1/2\pi n$. Putting the chronaxie $= 0.693k$ (Hill, 1936*b*, p. 442), we obtain

$$\text{chronaxie} = 0.110/n.$$

According to Coppée it is $0.33/n$. Coppée, however, did not use repetitive current pulses, but a single current, and we have found above (§ III) that this may have a considerable effect. If his observed rheobase was too high, say twice as high as it would have been for repetitive pulses, then his "isopotential current" also was twice as high and I^2/I_0^2 instead of being 2 was really 8. This would give $4\pi^2 k^2 n^2 = 7$, from which $0.693k = 0.29/n$. The last figure agrees approximately with the number he gives, viz., $0.33/n$. It is difficult, however, to believe that his rheobase can have been so much too great; some additional cause is required to explain the divergence. Possibly the wave form of his oscillator was not good enough. (Cf. his fig. 5, p. 245, 1934*a*.)

There is no particular significance in it, but it is interesting to note that the stimulus of minimum energy (per impulse started) is the same as Coppée's "isopotential current". The energy per stimulus (neglecting alterations of impedance with frequency) is proportional to I^2/n , i.e. (from equation (6)), to $(1/n + 4\pi^2 k^2 n)$. This is a minimum when $n = 1/2\pi k$ or $k = 1/2\pi n$ as we found above. Thus $k =$ (period of stimulating current of minimum energy)/ 2π . This may be compared with the cases of stimulation (1) by condenser discharges and (2) by constant current pulses: for (1), $k = RC$ for minimum energy; for (2), $k =$ (pulse duration for minimum energy)/1.26 (Hill, 1936*b*, p. 443).

V—THE EFFECT OF INTER-ELECTRODE DISTANCE ON THE I - n RELATION

The Effect on k and Rheobase—We have just shown that k is the same when determined (a) by condenser discharges, and (b) by alternating current. This might conceivably be due to chance. Temperature has a similar effect on k obtained by either method, but the effect of temperature is not specific. A more specific means of changing k is to alter the inter-electrode distance. It was found by Cardot and Laugier (1914) and Cardot (1914), and confirmed by others (see Hill, 1936*b*, p. 448) that reduction of interpolar length increases the rheobase and decreases the value of k . Experiments, therefore, were made to find out whether diminished interpolar length has the usual effects on rheobase and k , when these are obtained by alternating current stimulation.

For this purpose, the I - n relation was determined for two interpolar distances (about 2 and 25 mm. respectively), using either the electric response of the nerve (a constant small galvanometer deflexion, or

TABLE X

(a) EFFECT OF INTERELECTRODE DISTANCE ON k OF NERVE DETERMINED BY ALTERNATING CURRENT																Mean
Temperature, ° C.	24	24	20	23	22	23	26	23	20	20	20	6	21	23		
Electrode separation, mm.—																
Long	30	27	28	28	28	27	25	25	20	23	22	26	19.5	19.5	25	
Short	1.9	1.9	1.9	2.0	2.0	2.0	2.0	1.5	3.5	3.0	1.7	2.0	1.5	1.5	2	
Time factor k , msec.—																
Long	0.32	0.40	0.89	0.40	0.84	0.41	0.39	0.45	0.42	0.40	0.32	0.82	0.42	0.33	0.49	
Short	0.26	0.22	0.28	0.24	0.32	0.22	0.23	0.23	0.26	0.23	0.23	0.59	0.26	0.16	0.27	
The first eight experiments were made with muscle response as index; the remainder with nerve action current.																
(b) EFFECT OF INTERELECTRODE DISTANCE ON k AND λ DETERMINED BY ALTERNATING CURRENT																Mean
Temperature, ° C.	21	23.5	24.5	27.5	28.5	26	28.5	26.5	28	28.5	26	26.5				
Distance, mm.																
Long	19.5	19.5	22	22	22	15	15	22	22	22	25*	25*	22		22	
Short	1.5	1.5	2	2	2	2	1.5	1.8	2	2	2	2	1.9		1.9	
k , msec.																
Long	0.42	0.33	0.64	0.34	0.27	0.37	0.28	0.52	0.32	0.39	0.41	0.37	0.39		0.39	
Short	0.26	0.16	0.22	0.17	0.125	0.25	0.15	0.21	0.175	0.17	0.28	0.25	0.21		0.21	
λ , msec.																
Long	6.3	6.6	3.8	4.3	3.9	4.3	3.5	3.0	3.3	2.4	4.1	2.9	4.0		4.0	
Short	6.6	5.3	4.6	4.3	3.7	4.1	2.9	3.4	3.0	3.3	4.6	3.3	4.1		4.1	

* 8 mm. of far end crushed.

occasionally a constant fraction of maximal response determined at each frequency) or a small tetanic muscle response, as index of nerve excitation. Three stimulating electrodes were used, the "fixed" point being placed nearest to the muscle or to the lead-off electrodes. In these experiments it was impracticable to injure the nerve between the stimulating leads: impulses, therefore, originating from the two "variable" stimulating points could not be avoided.

Typical results are shown in fig. 6 and Table XI, and a summary in Table Xa and *b*. The threshold intensity at low frequencies was always higher for short interpolar length; the threshold ratio between small and great distances diminished with increasing frequency. The time factor k , determined, as described in § IV, from the I^2-n^2 relation in a low frequency range on the high side of the optimum, was always smaller for the short interpolar length. Using 2 mm. and 25 mm. distances respectively, the ratio k (long)/ k (short) averaged 1.85 in the experiments

TABLE XI

EXPERIMENT OF 4 APRIL, 1936, AT 23° C., 19.5 MM., AND 1.5 MM. INTERELECTRODE DISTANCES. SMALL ELECTRIC RESPONSE OF NERVE AS INDEX.

Frequency, per second	30	50	70	100	150	200	300	500
Threshold intensity, μ A. (r.m.s.)								
Long	2.9	2.6	2.4	2.4	2.4	2.5	2.8	3.5
Short	11.6	10.0	9.3	8.8	8.6	8.6	8.7	9.5
Frequency, per second	700	1000	1500	2000	3000	4000	5000	6000
Threshold intensity, μ A. (r.m.s.)								
Long	4.1	5.2	6.8	8.6	11.9	15.5	19.5	22.9
Short	10.2	11.7	13.1	14.9	18.5	23.4	28.3	33.0

EXPERIMENT OF 30 APRIL, 1935, AT 20° C., 20 MM., AND 3.5 MM. INTERELECTRODE DISTANCES. SMALL ELECTRIC RESPONSE OF NERVE AS INDEX

Frequency, per second	100	300	500	800	1000	1300
Threshold intensity, μ A. (r.m.s.)						
Long	1.22	1.58	1.97	2.67	3.19	3.89
Short	2.38	2.66	3.05	3.69	3.99	4.59
Frequency, per second	1600	2000	2500	3000	4000	5000
Threshold intensity, μ A. (r.m.s.)						
Long	4.73	5.79	7.31	8.89	12.2	16.4
Short	5.21	5.84	6.82	7.92	10.0	12.7

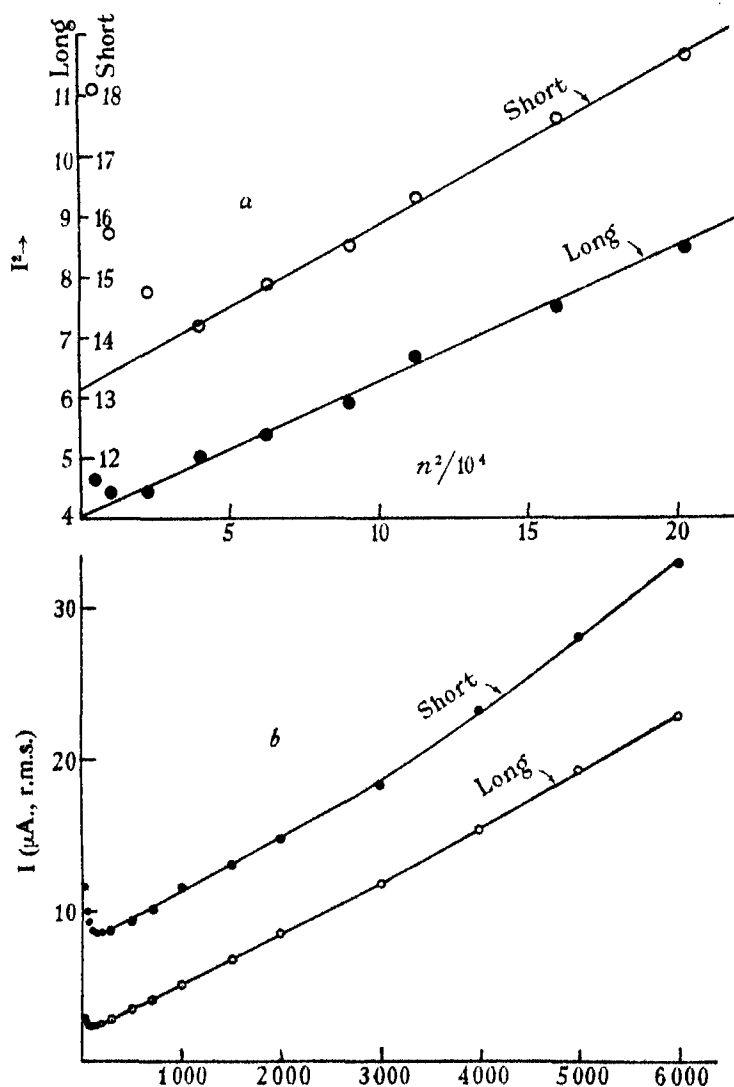


FIG. 6—Effect of interpolar length, in alternating current stimulation, on (a) the I^2-n^2 relation at lower frequencies, (b) the $I-n$ relation at higher frequencies. (a) 21 March, 1936: 26°C. ; interelectrode distances, 25 mm. and 2 mm.; small muscle tetanus as index. k (long) = 0.39 msec.; k (short) = 0.23 msec. (b) 4 April, 1936: 23.5°C. ; interelectrode distances, 19.5 mm. and 1.5 mm.; small constant nerve response as index.

of Table Xa; with 2 mm. and 20 mm. (means) it averaged 2.0 in those of Table Xb. Bouckaert found (Hill, 1936b, p. 449) that k determined by condenser discharges was about twice as great for 15–20 mm. as for about 1 mm. interpolar distance.

The curves obtained at small inter-electrode distance were apt to show certain deviations from the theoretical relation, as is the case also with condenser discharges. Using a threshold muscle response as index, a "double" curve was often found when fitting the theoretical log E -log RC curve (Hill, 1936b) to the observed values. The I^2-n^2 relation sometimes showed distinct curvature (the slope diminishing with increasing frequency) even at frequencies just beyond the optimum.

Occasionally the I - n curves for small and great interelectrode distance crossed at some intermediate frequency (see Table XI). In these cases, the ratio of threshold intensities for short and long interpolar length was less than 2 at the optimum frequency (usually being greater than 3) while at high frequencies I (short)/ I (long) became less than unity. When applying one-way pulses or one-way condenser discharges with a fixed stimulating cathode for all distances used, the corresponding phenomenon was never observed. With alternating current, however, one cannot avoid the possibility of exciting at both electrodes (since, for the present purpose, the variable electrode region could not be injured), and the variable electrode region may have different excitabilities for "short" and "long". If, by chance, the "variable" stimulating point for the small distance had a higher excitability than the two other points, the crossing of the I - n relation shown in Table XI is easily understood.

We conclude that the effects of interelectrode distance on rheobase and k , when these are determined by alternating current, are the same as when they are derived from condenser discharges: which adds to the conviction that the quantities obtained by the two methods are identical in nature.

The Effect on λ —In § VI below the value of λ determined by alternating current on the low side of the optimum frequency is shown to be the same as that derived from exponentially increasing current. The effect of electrode separation on the value of λ determined by exponentially increasing current has been examined by Schriever (1932) and by Solandt (1936a). Schriever measured what he called the "*Einschleichzeit*", which (Hill, 1936a) should bear a constant ratio to λ . In five experiments at 30 mm. and at 3 mm. respectively, the mean ratio of his times was 1.25 (1.12 to 1.35); the mean ratio of the "chronaxies" was 1.62 (1.45 to 1.73). Solandt in eight experiments with long (30 mm.) and with fairly short (4–6 mm.) distances found no regular difference in λ . In six

experiments with 30 mm. and 1–2 mm. the mean ratio λ (long)/ λ (short) was 1.3 (1.25 to 1.41). According to Bouckaert the ratio of the k 's should be about 2. The effect of interpolar length on λ is clearly far less than on k .

It was desirable to verify this conclusion with alternating current, and 12 experiments were made (Table Xb) in which both λ and k were determined from observed data using equations (5) and (6), (a) for an electrode separation of about 20 mm., and (b) for one of about 2 mm. The method was to employ three stimulating electrodes (two pairs) as described above, and to find the threshold I for a number of frequencies n , from 30 to about 1000 cycles per second. At each n , I was determined for short and for long interpolar distance, and when the series had been completed with increasing frequencies a reverse series was made, and the mean value of I at each n taken for the calculation. A minimal electric response was used as index of constant excitation.

As usual I^2 was plotted against $1/n^2$ and n^2 respectively, on the low and the high sides of the optimum frequency, and so λ and k determined. With the short interpolar distances, sometimes deviations from the linear relations occurred at the very low (30/sec.) and at the higher (> 500 /sec.) frequencies, in the sense that at these the observed points lay *below* the lines. These anomalous points were disregarded and the values of λ and k calculated from the good linear relations found over the rest of the range. If attention had been paid to them its effect would have been to make k still smaller, and λ larger, for short interpolar length, and so to emphasize the chief conclusion we shall draw, viz., that λ/k is increased by a reduction of interpolar length.

The results are given in Table Xb. At an average temperature of 26° C., the values of k for 20 and 2 mm. separation were respectively 0.39 and 0.21 msec., while the values of λ were 4.0 and 4.1 msec. Thus λ determined by alternating current is unaffected by interelectrode distance within the range 2–20 mm., while k is doubled. In the experiments of Table Xb the mean value of λ/k for 20 mm. distance is 10, for 2 mm. distance 20. This will be referred to in § VIII below.

The experiments of Table Xb were very consistent and left no doubt of the result. Experience has convinced us that of all methods of determining the time constant λ on rapidly accommodating nerve, the best is that based on equation (5) employing alternating current below the optimum frequency. This may be due partly to the fact, illustrated in fig. 1, that threshold excitation occurs with alternating current when the threshold is normal, i.e., in the just unaccommodated nerve. Whatever the reason, we are inclined to lay much more weight on the present results.

than on those of Solandt and of Schriever, and to attribute such effect of diminished interpolar length as they found with exponentially increasing currents, to some consistent complicating factor. The conclusion we draw is that, while k is considerably affected by diminished interpolar length, λ is almost, or altogether uninfluenced.

The effect of electrode separation in changing k without changing λ can be illustrated in another way, as in fig. 6. This represents the fourth experiment of Table Xb. The observed value of I , divided by an interpolated value of I_{op} , is plotted against $\log n$, for 22 and for 2 mm. separation respectively. For the 2 mm. the optimum frequency is 186 cycles

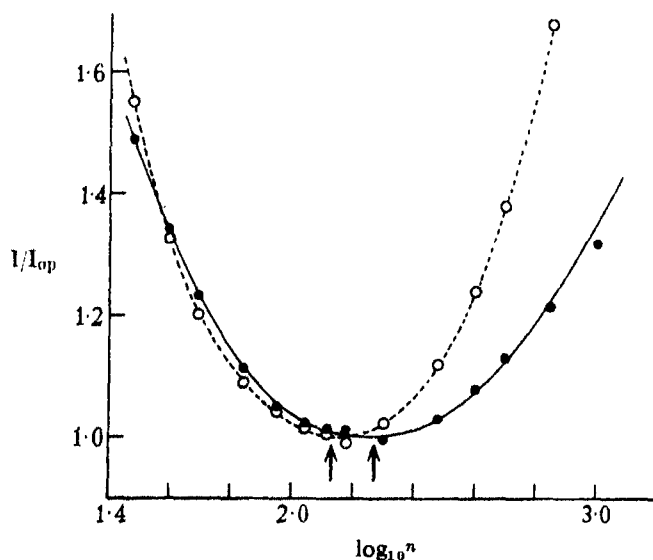


FIG. 7—Relation between threshold alternating current and frequency for short (full circles, full line) and for long (hollow circles, broken line) interelectrode distance, 2 mm. and 22 mm. respectively, 27.5° C. The threshold at the optimum frequency is taken as unity for plotting in each case; actually it was for 22 mm. 1.91 μ A, for 2 mm. 6.97 μ A. The arrows represent the optimum frequencies, 186 and 135 respectively.

per second; from this $k\lambda = 0.73 \times 10^{-6}$, agreeing with the product of k and λ of Table Xb. For the 20 mm., $n_{op} = 135$; hence $k\lambda = 1.39 \times 10^{-6}$, agreeing approximately with the product of k and λ from the table.

The difference of optimum frequency is immediately evident from fig. 7. So also is the difference of shape, which (fig. 2) depends on the value of k/λ . Comparison of fig. 7 and fig. 2 shows at once that k/λ is considerably greater for 22 mm. than for 2 mm. separation. A similar figure could be

drawn for each of the experiments of Table Xb. The increase of optimum frequency with diminished interpolar length is a sign that $k\lambda$ is less: the broadening of the curve, a sign that k/λ is less. This is only another expression of the fact that k is diminished while λ is little, if at all, affected.

According to Coppée (1935c, p. 32), the optimum frequency was not modified appreciably by bringing the electrodes nearer (0.1 mm. must surely mean 1.0 mm.); but the "isopotential frequency" and the "flatness" of the $I-n$ curve were increased. The former would mean that $k\lambda$ was unaltered; the latter that k/λ was diminished. This could be true only if k became less and λ proportionally greater with reduced electrode separation; the latter is contrary to our experience. Some disturbance probably affected Coppée's result, for he admits that with shorter inter-polar distance his $I-\log n$ curve lost its symmetry and became tilted.

The fact that the speed of accommodation is so little affected by distance between electrodes will be pertinent in any discussion of the physico-chemical nature of the process.

VI—THE COMPARISON OF λ CALCULATED FROM THE INTENSITY-FREQUENCY RELATION WITH ALTERNATING CURRENT, WITH λ CALCULATED FROM OBSERVATIONS WITH EXPONENTIALLY INCREASING CURRENTS

Another crucial test of the theory was to compare the value of λ calculated by equation (5) from experimental data with alternating current, with that deduced from the relation between threshold strength and time-constant of rise with exponentially increasing current. For the former, I^2 was plotted against $1/n^2$ on the low side of the optimum frequency, and λ was calculated from the linear relation obtained. (The procedure is illustrated in figs. 3b and fig. 9b.) For the latter, the method described by Solandt (1936a) was employed. The results have been given already by Solandt (1936b, Table IV). In order to obtain a high optimum frequency, and so to make the determination of λ by alternating current more accurate, the preparation was used at a rather high temperature (average 25.6° C.) and had previously been soaked for 1 to 5 hours in a Ringer's solution containing eight times the normal concentration of calcium. The value of λ , therefore, was small. In the experiments with alternating current a minimal steady tetanus of the muscle was taken as index of constant response in the nerve: in those with the exponentially rising currents, a minimal twitch was used. In 12 experiments the mean value of λ with alternating current was 4.6 msec., with exponential currents, 4.9 msec. The agreement is so good as to leave no reasonable doubt that the same quantity is measured by both methods.

VII—THE OPTIMUM FREQUENCY

In a large number of experiments extending over a year the optimum frequency for alternating current stimulation was determined in frogs' nerves under a variety of conditions. In most of these experiments the Muirhead oscillator was used, but in some, particularly those with a low optimum frequency, the photoelectric arrangement described in § II. In some experiments muscle response, in others nerve response, was used as index of constant excitation. In every case the optimum frequency was determined by plotting I against $\log n$ (see figs. 2 and 3 above).

The results are given in Table XII. They are divided into groups according to the temperature and according to whether the Ca-concentration of the Ringer's solution in which the preparation was soaked was normal or high. If the three mean results with normal Ringer's fluid are plotted, $\log n_{op}$ against the temperature, an approximately straight line is obtained (fig. 8) from which a mean value of the optimum frequency for

TABLE XII—OPTIMUM FREQUENCIES, CYCLES PER SECOND

I—After soaking in normal Ringer's solution—

- (a) At 6° C.: 10, 19, 21, 24, 30; mean 21.
- (b) At room temperature, 16–22° C., average about 18½° C.: 40, 40, 42, 45, 48, 50, 54, 54, 54, 59, 60, 62, 63, 63, 69, 75, 77, 77, 80, 81, 81, 85; mean 62.
- (c) At 24 to 31° C., average 28° C.: 115, 123, 123, 128, 136, 140, 140, 144, 155, 170, 186, 195, 206; mean 151.

II—After soaking in Ringer's solution containing high Ca (4 to 10 times normal)—

- (b) At room temperature, 17–23° C., average 19½° C.: 76, 93, 95, 95, 102, 103; mean 94.
- (c) At 30–34° C., average 31½° C.: 178, 191, 210, 224, 245, 245, 270; mean 223.

the particular frogs and Ringer's solution used can be read off at any temperature. At 10°, 20°, and 30° C., respectively these interpolated values are 29, 71, and 186 cycles per second. An approximate Q_{10} is 2.5.

The effect of temperature on the optimum frequency was shown by v. Kries (1884) who found that in "warm" nerve it might be more than 200, in "cold" nerve less than 50, by Achelis (1930) and by Coppée (1934c, p. 40). According to the latter the I - $\log n$ curve is simply translated to the right, i.e., without change of shape, by a rise of temperature. This would mean that λ/k was unaffected by temperature. We have no direct evidence on this point in a single preparation at different temperatures, but in our experience the lower values of λ/k were far more frequent at the higher temperatures.

The two mean results with high calcium are shown in the same figure. At room temperature the effect of calcium in raising the optimum frequency is considerable. Two special experiments were made to verify this. In one, a preparation at 21° C., after soaking 2 hours in normal Ringer's fluid, gave $n_{op} = 40$; after a further 5½ hours in 10 times normal Ca it gave $n_{op} = 95$ at 23° C. In the other at 22° C., after soaking 4½ hours in normal Ringer's fluid, n_{op} was 77; after a further 4 hours in 10 times normal Ca it was 102 at 23° C. At about 30° C., however, the effect of high calcium appears to be negligible. According to Solandt (1936*a*), a high Ca concentration considerably diminishes λ , and since

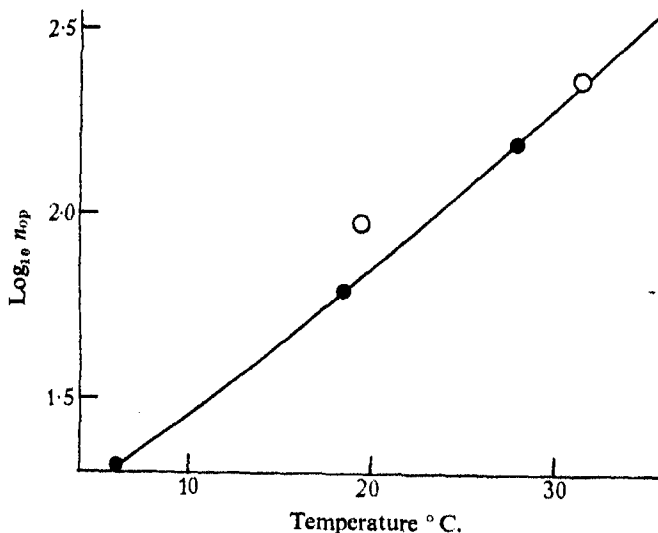


FIG. 8—Effect of temperature on the optimum frequency in alternating current stimulation. Mean results of Table XII. ● normal Ringer's solution; ○ nerve treated with high Ca.

(equation (2)) n_{op} is inversely proportional to $\sqrt{\lambda}$, n_{op} is increased. At a high temperature, however, λ is already so small that a high Ca apparently does not diminish it further.

A value of $n_{op} = 62$ for room temperature gives, from equation (2), $k\lambda = 6.6 \times 10^{-6}$. A mean value of k for this temperature is about 0.4 msec., so that the corresponding value of λ should be about 16 msec. This is well within the normal range found by Solandt (1936*a*) at room temperature with exponential currents. The value of $n_{op} = 94$ for room temperature with high Ca-nerves corresponds to $k\lambda = 2.9 \times 10^{-6}$, which with $k = 0.4$ msec. requires $\lambda = 7.2$ msec. This is in the range of small values of λ found by Solandt for nerves with high Ca. A value of

$n_{op} = 151$ at 28° C. in normal nerve corresponds to $k\lambda = 1.11 \times 10^{-6}$ which with $k = 0.25$ msec. requires $\lambda = 4.4$ msec. A value of $n_{op} = 223$ at $31\frac{1}{2}^{\circ}$ C. in high Ca nerves corresponds to $k\lambda = 0.51 \times 10^{-6}$, which with $k = 0.2$ msec. requires $k = 2.5$ msec. At the other extreme, a value of $n_{op} = 21$ at 6° C. corresponds to $k\lambda = 57 \times 10^{-6}$, which with $k = 1.0$ msec. requires $\lambda = 57$ msec. These are all values which experience has led one to expect as usual.

The behaviour, therefore, of the optimum frequency, with respect to changes of temperature and of calcium concentration, is what we should predict from the known effects of these on k and λ , and its absolute value corresponds to reasonable values of those quantities.

An attempt was made to obtain a very low optimum frequency by increasing the value of λ by depriving the nerve of ionized calcium. The best way of decreasing the Ca-ion concentration is to soak the nerve in an appropriate citrate solution (soaking a nerve in low calcium solution takes a very long time), and we have found a clear relation to exist between the value of λ observed by exponential currents and the Ca-ion concentration calculated from the formulae of Hastings, McLean, Eichelberger, Hall, and Da Costa (1934); this will be described in a later paper.

A nerve was soaked in citrate-Ringer's solution and then stimulated with the photoelectric arrangement described in § II. The latter was necessary since the optimum frequency expected was very low. The results were very complicated. There were signs of the usual $I-n$ relation, but superimposed on this were effects which we could only attribute to repetitive response of the nerve to each cathodic half-wave. Katz (1936*b*, *c*) has shown that when accommodation is very slow a long series of action currents may occur when a constant current is passed through a medullated nerve, and we imagine that something similar must have happened with the long-lasting waves of the alternating current at low frequency. It proved impossible to determine the optimum frequency with any certainty, and the experiments were not continued.

An attempt was made also to find the optimum frequency of the human ulnar nerve. This proved to be below the range in which the wave form of the oscillator, with its amplifier, could be trusted, and the photoelectric arrangement did not give enough current. We suspect, moreover, that repetitive response of the slowly accommodating nerve was affecting the result. According to Solandt (1936*a*), a normal value of λ in human ulnar nerve is 70 msec. This, on the analogy of frog's nerve, is sufficient to give repetitive response to a constant current of rather low intensity. We had embarked on the experiment without making a calculation which would have shown it to be useless. With $k = 0.35$ msec.

and $\lambda = 70$ msec. the calculated value of the optimum frequency is $n_{op} = 32$, and with such a large value (200) of λ/k it is necessary (fig. 2) to go far below the optimum before the rise of threshold in that region can be measured. With an apparatus capable of giving an alternating current of several milliamperes at (say) 50 volts, and of pure enough wave form from (say) 2 to 500 cycles per second, the phenomena of optimum frequency could probably be demonstrated in human nerve—but only provided that, at the lower frequencies, repetitive response to each cycle did not spoil the result. Such apparatus was not available and would have had to be specially constructed, and, moreover, the hope of avoiding repetitive response was not great, so the experiments were discontinued.

VIII—THE RELATION BETWEEN THE TWO TIME-CONSTANTS OF EXCITATION

In fig. 2 it is shown that the theoretical form of the I -log n curves, on either side of the optimum frequency, depends on the ratio of the two time-constants, being more pointed (rising more steeply on either side of the optimum) the less the value of λ/k . Coppée (1934c, p. 16) was aware of this variation, and used as a measure of the flatness ("aplatissement") of his curves the width of the I - n (not I -log n) relation at twice the minimum threshold height. Let n_1 and n_2 be the frequencies for threshold $2I_{op}$, then Coppée measured the flatness of his curves by the factor $a = (n_1 - n_2)/n_{op}$. He states that for the frog's sciatic nerve a varies from 3.5 to 8, for the non-neural part of the sartorius of frog and toad from 11 to 13, for the foot of a snail from 3.5 to 8.

Putting $I/I_{op} = 2$ in equation (4'), and solving for n/n_{op} we find

$$a = (n_1 - n_2)/n_{op} = \sqrt{3(\lambda/k + k/\lambda) - 6}.$$

From this the following values of a can be calculated:—

λ/k	2	3	5	10	20	40	60	100
a	3.67	4	4.65	6.02	8.14	11.22	13.63	17.5

The values of a given by Coppée correspond to the following values of λ/k :—

Frog's sciatic nerve	$\lambda/k = 1.6 - 19.5$
Sartorius	$\lambda/k = 38.5 - 54$
Snail's foot	$\lambda/k = 1.6 - 19.5$

The variation is wide. The higher figure for nerve is well within our normal range; the lower figure, however, is completely outside it: we have never observed a value of λ/k of less than 7.3. Moreover, the results

given in Coppée's Table I for frogs' nerves allow the value of k to be calculated from the chronaxie ($= 0.693k$), and the value of $k\lambda$ to be calculated from the optimum frequency (equation (2) above), from which λ , and so λ/k , can be determined. The results are given in Table XIII.

TABLE XIII

k , msec.	0.23	0.245	0.36	0.29	0.24	0.22	0.25	0.245	0.22
λ , msec.	3.4	6.1	7.0	8.0	4.7	10.6	6.5	4.9	2.6
λ/k	15	25	19	28	20	48	26	20	12

The values of λ/k lie well within our normal range: not one of them approximates to the 1.6 calculated from Coppée's a . We suspect that some error must have affected the latter. Further, Coppée (1936c, p. 18) states that in his experiments the period corresponding to the optimum frequency was 30–60 times the chronaxie: from this it can be calculated that λ/k lay between 11 and 44. In any case, all his results confirm Solandt's (1936a) conclusion that the value of λ/k is highly variable.

That λ/k is variable under the conditions of the present experiments with alternating current is shown by the fact that we have obtained values of λ/k from 7.3 to 45. It would be easy to get larger values than 45, but in order to have a high optimum frequency we have intentionally worked at a high temperature and very often with a nerve previously soaked in a high Ca-solution, both of which tend to give a low value of λ/k . In normal (spring) nerves, after soaking for a few hours in normal Ringer's fluid, we have found values at about 28° C., ranging from 10 to 27: after soaking for 24 hours, one of 45. In high-Ca nerves at about 32° C. we have found values from 7.3 to 20. On one occasion at 23° C. a high-Ca nerve gave $\lambda/k = 34$. A normal nerve at 17° C. gave a value as low as 17.

High values of λ/k at low temperature mean a low optimum frequency and a nerve accommodating slowly, conditions which practically ensure repetitive response to each cathodic half-wave on the low side of the optimum frequency. With a large value of λ/k the curves of fig. 2 are so blunt that one would have to go to very low frequencies to obtain any accuracy in the determination of λ . It is unprofitable, therefore, to work with alternating current on frogs' nerves in this region of very low frequency: which is the reason why we have not obtained values of λ/k much larger than we have actually found. Even within our range, however, of values of λ/k the variation is so wide (more than sixfold) that it is impossible to maintain that (in Lapicque's words, quoted by Fabre, 1934) "*Toute constante de temps obtenue sur un organe excitable au moyen d'un*

courant de forme quelconque est proportionnelle à la chronaxie de cet organe". Monnier in his theory (1934) assumed λ/k (his τ_2/τ_1) to be constant and equal to 6. Fabre also originally followed authority in supposing that his "*constante linéaire*" is proportional to the chronaxie, and gave a formula connecting them; later (1931, 1934) he came to the conclusion that the former can vary independently of the latter. In this we confirm him: λ/k in frog's nerve, according to our experience, may have any value between about 7 and almost infinity.

IX—THE AGREEMENT OF THE I - n RELATION WITH THEORY

Many experiments have been made in which the relation between threshold intensity and frequency was determined. A few of them are recorded in Table XIV. These have been chosen to represent several different conditions. In the first two a minimal steady muscle response was used

TABLE XIV—THE RELATION BETWEEN I AND n ON BOTH SIDES OF THE OPTIMUM FREQUENCY

Experiment of 9 January, 1936—Muscle nerve preparation of Hungarian *Rana esculenta* after 3 hours 10 minutes in Ringer's solution with $6 \times \text{Ca}$, at 32.2°C . Minimal tetanic response of muscle: clear sharp index, no doubt of reading. Nerve 48,000 ohms, external resistance 952,000 ohms. Series from 30 to 1300 cycles per second and reverse; mean of two readings at each frequency. Note that the thresholds are high because of the calcium.

Cycles per second	30	50	70	100	150	225
Threshold, $\mu\text{A. (r.m.s.)}$	8.55	6.1	5.25	4.75	4.45	4.35
Cycles per second	350	500	700	900	1100	1300
Threshold, $\mu\text{A. (r.m.s.)}$	4.50	4.75	5.3	5.95	6.6	7.25

In fig. 3*b* above I^2 is plotted against n^2 for the 9 highest frequencies, and I^2 against $1/n^2$ for the 8 lowest frequencies. The two relations approximate to straight lines, on the higher and the lower sides respectively of the optimum frequency. As that frequency is approached both relations bend up. The straight portions allow one to read off the values of k and λ respectively, and also the value of I_0 .

In fig. 3*a* I is plotted against $\log_{10} n$, and a theoretical curve (equation (1)) is drawn with the constants, $I_0 = 4.10$, $k = 0.1815 \text{ msec.}$, $\lambda = 2.91 \text{ msec.}$ The fit is good. From equations (3) and (2) $I_{op} = 4.36$ and $n_{op} = 219$; $\lambda/k = 16$. An exactly similar and equally good experiment was made on the opposite preparation of the same frog. It has been in the high-Ca solution for $1\frac{1}{2}$ hours only, and the temperature was 31°C . n_{op} was 210 cycles per second; I_{op} was $2.6 \mu\text{A. (r.m.s.)}$; k was 0.204 msec. and λ was 2.82 msec.

Experiment of 17 January, 1936—Muscle nerve preparation in normal Ringer's fluid 2 hours, then in chamber $2\frac{1}{2}$ hours at 28°C . Minimal tetanic response of muscle as index of excitation. Series from 30 to 1000 cycles per second and reverse; mean of two readings at each frequency.

TABLE XIV—(continued)

Cycles per second	30	50	80	130	200
Threshold, $\mu\text{A. (r.m.s.)}$	2.39	1.89	1.67	1.58	1.59
Cycles per second	300	400	600	800	1000
Threshold, $\mu\text{A. (r.m.s.)}$	1.745	1.95	2.38	2.91	3.43

In fig. 9*b* I^2 is plotted against n^2 for the 8 highest frequencies, and I^2 against $1/n^2$ for the 6 lowest frequencies. The two relations approximate to straight lines, on the higher and the lower sides respectively of the optimum frequency. As that frequency is approached both relations bend up. The straight portions allow one to read off the values of k and λ respectively, and also the value of I_0 .

In fig. 9*a* I is plotted against $\log_{10} n$, and a theoretical curve (equation (1)) is drawn with the constants, $I_0 = 1.46$, $k = 0.355$ msec., $\lambda = 3.97$ msec. The fit is good. From equations (3) and (2), $I_{op} = 1.583$ and $n_{op} = 138$; $\lambda/k = 11.2$.

Experiment of 5 April, 1935—Sciatic nerve of Hungarian *Rana esculenta*, soaked for a few hours in high-Ca Ringer's solution, at room temperature (about 18° C.). Constant fraction of maximal action current of nerve, observed with sensitive galvanometer. Readings of I , in arbitrary units, in order given below. Stimulation with photo-cell arrangement. The calculated values are for $I_0 = 53$, $k = 0.47$ msec., $\lambda = 8.2$ msec. From equations (3) and (2), $I_{op} = 56$, $n_{op} = 81$; $\lambda/k = 17.5$.

Cycles per second	5.66	9.83	34.3	79	148	266
Threshold—						
Observed	182	126	61	58	58	67½
Calculated	189	117	61	56	58	66
Cycles per second	146	75	59	30.4	6.1	
Threshold—						
Observed	61	60	58	63	167	
Calculated	58	56	57	63	176	

Experiment of 27 March, 1936—Sciatic nerve soaked several hours in normal Ringer's solution, at 28° C. Minimal action current of nerve observed with sensitive galvanometer. Series from 25 to 1000 cycles per second and reverse; mean of two readings at each frequency. By plotting I^2 against n^2 and $1/n^2$ respectively, as in figs. 3 and 9, k and λ were determined, and the values of I calculated from equation (1) with the following constants: $k = 0.266$ msec.; $\lambda = 2.52$ msec.; $I_0 = 1.31$. From equations (3) and (2), $I_{op} = 1.445$ and $n_{op} = 193$; $\lambda/k = 9.5$.

Cycles per second	25	30	40	50	60	70	90	110	130
Threshold I , $\mu\text{A. (r.m.s.)}$ —									
Observed	3.60	3.12	2.45	2.11	1.88	1.76	1.61	1.50	1.50
Calculated	3.53	3.04	2.44	2.11	1.91	1.78	1.61	1.53	1.49
Cycles per second	150	200	250	300	400	500	700	1000	
Threshold I , $\mu\text{A. (r.m.s.)}$ —									
Observed	1.445	1.445	1.455	1.48	1.57	1.73	2.00	2.48	
Calculated	1.465	1.445	1.47	1.50	1.60	1.73	2.03	2.57	

The agreement between calculated and observed is good, except at 1000 cycles per second.

TABLE XIV—(continued)

Experiment of 28 March, 1936—Sciatic nerve soaked for 24 hours in normal Ringer's solution (14 hours at 6° C.); experiment made at 28° C. Minimal action current of nerve observed with sensitive galvanometer. Series from 25 to 700 cycles per second and reverse; mean of two readings at each frequency. By plotting I^2 against n^2 and $1/n^2$ respectively, as in figs. 3 and 9, k and λ were determined, and the values of I calculated from equation (1) with the following constants: $k = 0.170$ msec.; $\lambda = 7.6$ msec.; $I_0 = 2.72$. From equations (3) and (2), $I_{op} = 2.78$ and $n_{op} = 140$; $\lambda/k = 44.7$.

Cycles per second	25	30	40	50	60	70	80	110
Threshold I , μA . (r.m.s.)—								
Observed	3.56	3.26	3.09	2.95	2.90	2.85	2.80	2.76
Calculated	3.55	3.32	3.07	2.95	2.88	2.85	2.79	2.78
Cycles per second	130	150	200	250	300	400	500	700
Threshold I , μA . (r.m.s.)—								
Observed	2.76	2.76	2.80	2.81	2.83	2.98	3.09	3.48
Calculated	2.78	2.78	2.79	2.82	2.86	2.96	3.08	3.40

The agreement between calculated and observed is good. *Note*—This is the opposite nerve to that of the preceding experiment (27 March); λ/k had increased from 9.5 to 44.7.

as index of constant excitation of the nerve: in the last three the action current of the nerve itself, recorded with a sensitive galvanometer. Of the first two, one was at 32° C., with a preparation which had been soaked in a Ringer's solution with six times the normal Ca; the other was at 28° C., with a preparation soaked in normal Ringer's fluid. Of the other three, one was at 18° C., with a nerve soaked in high-Ca; the optimum frequency being low, the photoelectric arrangement was used to provide the stimulating current. Both of the others were at 28° C., the first after a few hours and the second (on the opposite nerve) after 24 hours' soaking in normal Ringer's solution. In the first two experiments the observed threshold I is plotted against $\log n$ (figs. 3 and 9) and a *calculated* curve, drawn from equation (1) and the constants given in Table XIV, is drawn through the points. In the last three experiments the observed I is tabulated against n , together with a calculated I obtained from equation (1) and the constants given.

The agreement of calculated with observed is, in every case, good. Equation (1), therefore, correctly expresses the relation between threshold and frequency; and we have already shown (§§ IV and VI) that the values of k and λ , necessary to obtain the agreement, are the same as can be found by other experimental methods.

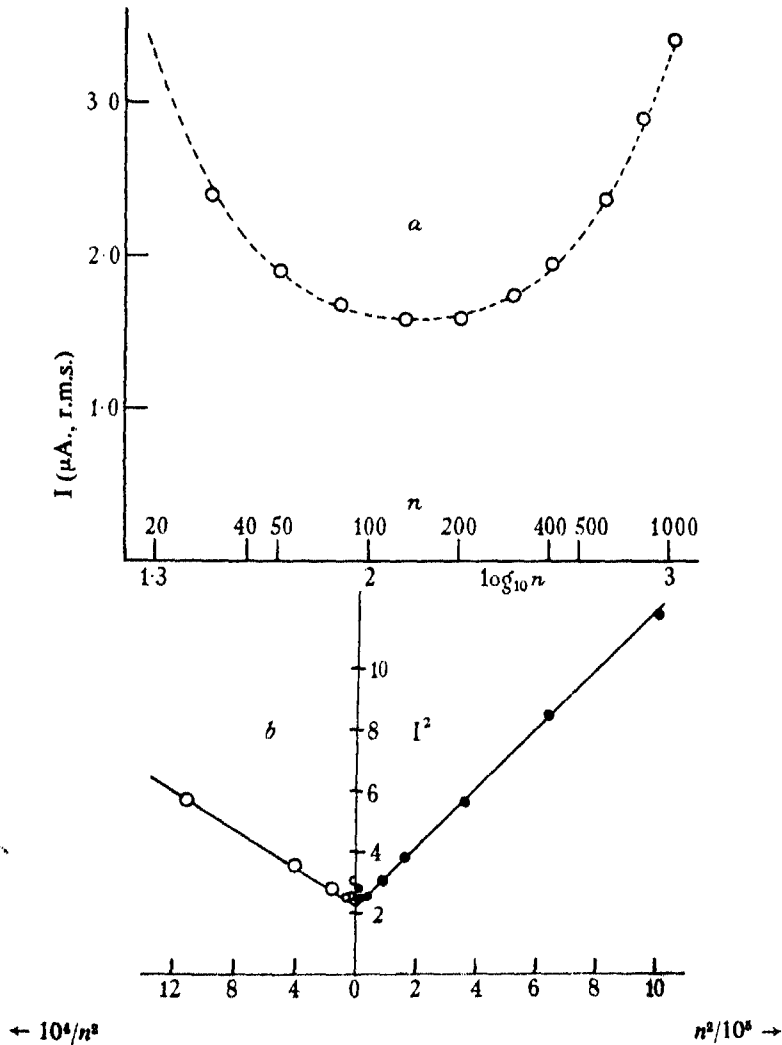


FIG. 9—Experiment of 17 January, 1936; Table XIV. Nerve treated with normal Ringer's solution, at 28°C . Muscle response as index. (a) I plotted against $\log_{10} n$, and a theoretical curve (from equation (1)) with the constants given in Table XIV. (b) I^2 plotted against (to the right) n^2 , and (to the left) $1/n^2$, showing linear relations on the high and the low sides respectively of the optimum frequency.

X—THE INITIAL IMPULSE DEPENDING ON THE PHASE AT WHICH THE ALTERNATING CURRENT IS MADE

Equations (1) to (6) above and the curves in fig. 1 all refer to a steady condition of repeated excitation, once to each cycle at the electrode considered. It was shown, however (Hill, 1936a, pp. 340, 342), that, when the current is first made, two extra exponential terms appear in the equations, depending on the phase at which the current begins. The experimental evidence for these will now be considered.

The *first* of them occurs in the expression for the "local potential" V . It dies out very rapidly. Its effect may be either to raise, or to lower, the local potential, according to the phase, but its decay is so quick that, owing to the refractory period, it cannot, under the most favourable circumstances, add to the excitatory effect of the current for more than one response of the nerve. The effect is greatest at high frequencies and when the current starts at phase zero, *i.e.*, at the beginning of a wave. When the index of successful excitation is a small steady action-current deflexion of a galvanometer its occurrence will not be noticed. When, however, the index is a muscle response, a single twitch may occur at a current intensity considerably below (theoretically, at high frequency, 50% of) that necessary for a maintained contraction; since this is a random effect, depending on the phase of making the current, it may, unless one is aware of it, give a considerable "scatter" to one's results. The only safe procedure is to take a minimal tetanic response as index of successful excitation and to neglect the twitch.

When using muscle response as index we have always noted the casual appearance of this initial twitch (von Kries's *Anfangszuckung*) at the higher frequencies. The phenomenon is not new and has been referred to by various authors (for references see Hill, 1936a, p. 341, and § XI below). Coppée (1934c) described it in detail and showed how to avoid it by progressive increase of the current to its threshold value.

The nature of this initial response at high frequency is easy to appreciate physically with the aid of fig. 1. At low frequency the local potential V follows the current practically without phase lag: its maximum cannot be made appreciably greater by beginning the current at any special phase. At high frequency V lags considerably behind the current, at very high frequency by $\pi/2$. The cathodic half of the wave, therefore, has to neutralize the effect of the anodic half before V begins to rise above V_0 . If the cathodic current begins at, or near, phase zero the neutralization has not to occur, and V rises higher. This happens to a considerable

extent only with the first cathodic half-wave, so that the excitatory effect of the current is appreciably enhanced only for one wave.

The *second* exponential term occurs in the threshold U . It dies out less rapidly than the first term, since it involves $e^{-t/\lambda}$ instead of $e^{-t/k}$. Its effect is predominant at low frequencies. Its mathematical expression is complex and need not be given here; it is easier to regard the matter physically. If an alternating current of low frequency begins to run through a nerve at the crest of a wave, *i.e.*, at phase $\pi/2$, it will be nearly as effective, *qua* stimulus, as a constant current of the same strength. It will be much more effective than the same current starting at phase zero, which will act as a slowly increasing current allowing the threshold time to rise. Thus with frequencies below the optimum, the twitch threshold—which is practically the constant current threshold if the phase $\pi/2$ is hit off approximately—will be nearly constant, while the steady tetanus threshold will be greater the lower the frequency.

If, therefore, one “makes” an alternating current of low frequency at random phase through a nerve, and takes a single twitch as index of response, the crest threshold will be found to vary casually, from the high level corresponding to the steady tetanus at that frequency to a low level approximately equal to that for a constant current. The only safe procedure is, as with high frequency, to take a minimal tetanic response as index of successful excitation and to neglect the twitch. The facts, without accompanying theory, have been admirably recorded by Coppée (1934c, pp. 10, 11). In his fig. 3 he correctly shows the I -log n relation, for sudden make of current and twitch response, on the low side of the optimum frequency, as nearly a straight line at rheobasic level.

The most exact of our experiments on the I - n relation were made with a very small steady action current of the nerve as index of constant excitation. With this, the make response is not noticed. Many, however, of our experiments were made with minimal muscle contraction as index, and in these, since we did not employ means (as Coppée did) either of increasing the current gradually or of starting it at phase 0 or π , we always noted, at low frequencies, the casual appearance of the initial twitch at a lower threshold than the steady response. A very simple means was found of avoiding it, *viz.*, to open the short circuit key K (fig. 4) very gently, instead of sharply, to make the current through the nerve. It may be that in this way the current increases gradually to its full value, or starts at phase zero. The muscle is saved from unnecessary stimulation, and the readings are made sharper, if this simple precaution is adopted. The only response observed is then usually the steady tetanus.

XI—THE INTENSITY-FREQUENCY RELATION AT HIGHER FREQUENCIES

In § IX above equation (1) has been shown to apply rather accurately, within certain limits of frequency, to stimulation of the sciatic nerve of *Rana esculenta* by alternating current. We shall now consider the limits of its application.

At low frequency the limiting factor seems to be the occurrence of repetitive response to each cathodic half-wave of the current; and with nerves accommodating slowly (with large λ) this means that the I - n relation cannot be determined accurately very far along its upward branch to the left. This will prevent the useful application of very low frequency current to slowly accommodating nerves, such as those of mammals or crustacea.

The upper limit to equation (1) appears to be set by the factors which come into play as the period of the current approaches the refractory period. If muscle response be used as index of excitation a further difficulty arises, at higher frequencies, from Wedensky inhibition. We have seen in § X that the twitch response is bound to yield irregular results depending on the phase of making the current; on the other hand, it is almost impossible to obtain a steady tetanic response of a muscle to high frequency stimulation of its nerve. We have made many attempts, using a muscle nerve preparation, to determine the I - n relation at higher frequencies, but have been forced to conclude that in this region a threshold steady response of the muscle is not a sufficiently objective index of nerve excitation to give the desired accuracy. There is, in fact, no steady response, but a rapidly declining one, and the decision as to what is a tetanic response and what a twitch depends too much on personal judgment. The only safe method is to avoid the neuromuscular junction altogether, and to work with a threshold response of the nerve itself. All our significant experiments, at higher frequencies, have used as index a very small action current response of the nerve, recorded with a sensitive galvanometer.

In a number of experiments the relation between I^2 and n^2 was determined beyond the range within which it is linear. It is not possible to say very exactly at what frequency the linearity ends; the curve gradually bends away from the straight line, nearly always in a downward direction (fig. 10). Roughly, however, an upper limit to the linear relation can be stated, and for 39 experiments is given in Table XV. Neglecting two interesting and anomalous cases of a very extended relation (to 2000 and 5000 cycles per second), the results can be divided up into four groups, as in the second part of the table. There is clearly an upward trend with

temperature. At 20° C., the upper limit, on the average, would be about 500 cycles per second; at 30° C., about 850. The former corresponds to a period of 2 msec., the latter to one of about 1·2 msec., quantities inside the relative refractory period and approaching the absolute refractory period of the nerve.

The usual type of deviation at higher frequencies from the linear I^2-n^2 relation, a rather exaggerated case for illustration, is given in curve (a), fig. 10. Here at 25·5° C., the I^2-n^2 line is not straight beyond 500 cycles

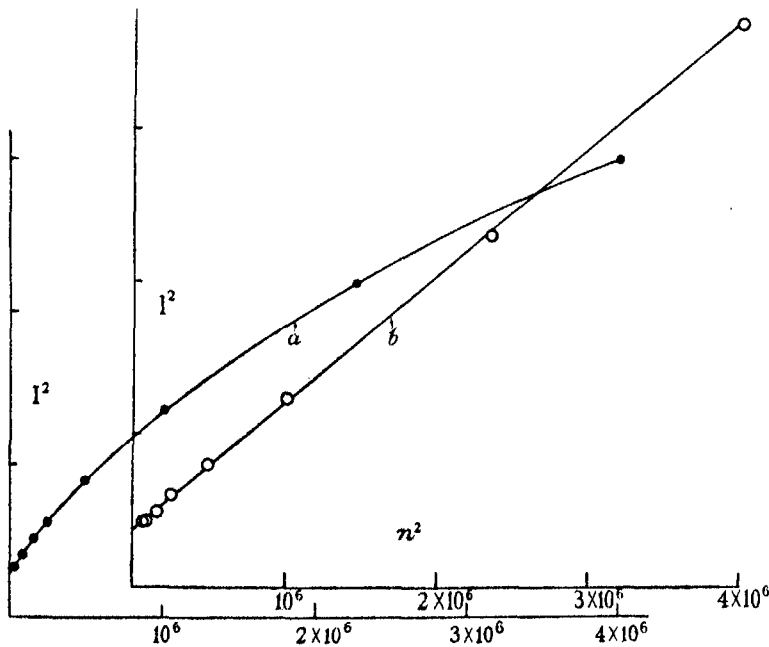


FIG. 10—The I^2-n^2 relation for threshold stimulation of frog's nerve by alternating current at higher frequency: minimal electric response as index of excitation. (a) 23 March, 1936; 25·5° C., $k = 0\cdot375$ msec.; (b) 27 March, 1936; 28° C., $k = 0\cdot245$ msec.

per second. For contrast, an unusual case at 28° C. is shown in fig. 10, curve (b). Here the line is straight to beyond 2000 cycles per second. Usually, however, the relations discussed in the earlier part of this paper apply only up to 400–1000 cycles per second according to the temperature; within that range they do apply, and rather accurately. We can give no convincing explanation of the form of the curve usually observed at higher frequencies, but it may be of importance that Harris, Rosenberg, and Sager (1936) found analogous deviations from a linear I^2-n^2 relation at

TABLE XV—UPPER LIMIT OF LINEAR RELATION OBSERVED BETWEEN I^2 AND n^2

(a)		16	17	17	18	18	18	18	19	19	20	20	21	22	22	22	23	23	23	26	27
Temperature, ° C. . .																					
Limiting frequency . .	450	350	500	350	500	530	700	350	500	400	2000	600	600	500	600	700	700	1000	500	600	
Temperature, ° C. . .		27	28	28	28	28	29	29	29	30	30	30	30	31	31	31	31	31	32	34	
Limiting frequency . .	700	500	800	900	1000	5000	900	1000	1000	500	500	850	750	900	1000	1000	1000	1100	1000		
•	(b)	Mean temperature, ° C.....																			
											18		22½		28		31				
		Mean limiting frequency									470		630		820		860				

higher frequencies when using, as index, not response but the attainment of a constant electrotonic potential.

For higher frequencies the plotting of I^2 against n^2 is not satisfactory, because if the higher range is properly represented the lower range is too cramped. A better graphical representation of the results is given at higher frequencies by plotting I against n . In fig. 11 four typical experiments are shown. The index of excitation was a very small steady electric response. In all four the I - n relation is approximately, in none is it

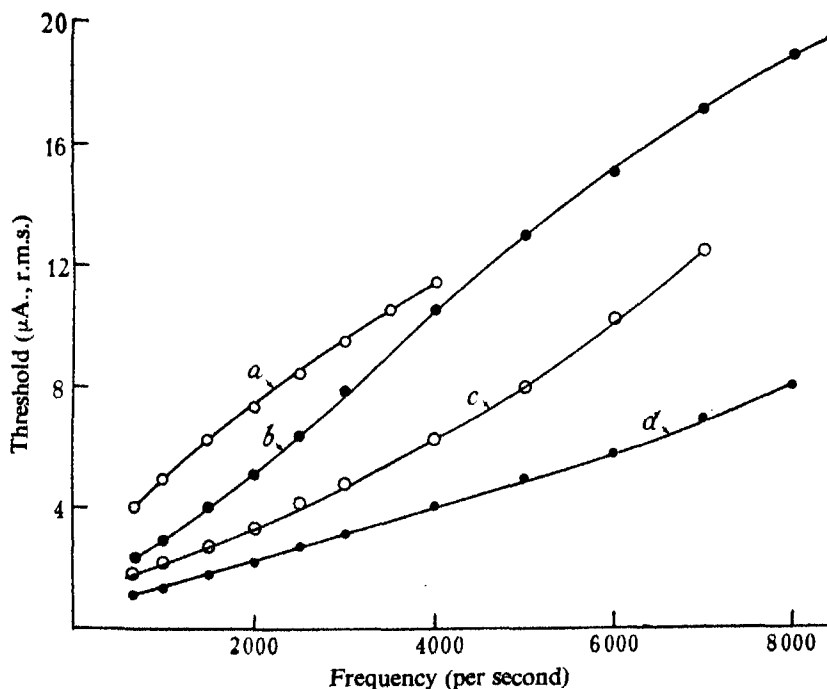


FIG. 11—Relation between I and n for threshold stimulation of frog's nerve by alternating current at higher frequency. Minimal electric response of nerve as index of excitation. (a) 23 March, 1936, 24.5° C.; (b) 27 March, 1936, 28° C., see Table XIV; (c) 28 March, 1936, 28° C., see Table XIV (I plotted on half the scale, to avoid confusion); (d) 27 March, 1936, 24° C. (I plotted on one-third the scale, to avoid confusion).

quite, linear. The deviation from a straight line is variable in either direction from one experiment to another. If by chance an exactly linear relation is found, no special significance need be attached to it.

At a low temperature, *e.g.*, at 8° C., or below, regular results of another type are obtained (fig. 12). The I - n relations bend upwards, the

threshold rising more than in proportion to the frequency. This effect is possibly due, at least in part, to a technical cause. The smallest steady response of the nerve which it is safe to take as an index of constant excitation is 1–2 mm., on the galvanometer scale. At higher frequencies (and 1000/sec. is a high frequency for a nerve at 6° C.) the response is not well maintained owing to the onset of the failure described by Bugnard and Hill (1935*b*, *c*); moreover, the maximal response, so far as it is possible to measure it, appears to be considerably less at high frequency than at low. For both reasons a steady response of 1–2 mm. requires a stimulus which may be more than the real threshold by an amount which

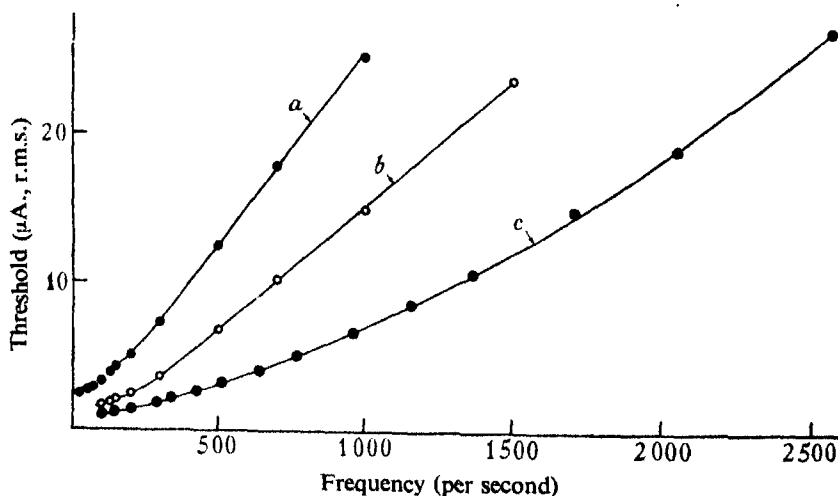


FIG. 12—Relation between I and n as in fig. 11 but at lower temperature, (a) 2 April, 1936, 6° C. (I plotted on three times the scale, to avoid confusion); (b) 1 April, 1936, 5° C.; (c) 21 December, 1934, 6° C. (I on arbitrary scale).

is greater at higher frequency. This, as a matter of fact, was the effect which, on the basis of the work of Bugnard and Hill, we had expected.

In studying nerve excitation by high-frequency alternating current of sine-wave form we have to take account of several different factors:

- (a) the influence of frequency of excitation as such;
- (b) the smaller excitatory effect of each cathodic half-wave as its duration is reduced;
- (c) the change of current distribution inside the structure of the nerve, or fibre, with alteration of frequency, due to electrical capacity either normally existing or produced by polarization at surfaces or membranes;

- (d) possibly depending on (c) a change over from the excitation of one type or group of fibres to another type or group, as the current distribution alters with frequency.

Of these factors (b) alone is taken into account in deducing equations (1) to (6) above. (a) was examined in detail by Bugnard and Hill (1935a, b, c) for maximal stimulation, using short condenser discharges of constant form but of variable frequency. (c) cannot be properly discussed until we have more definite information about the site and properties of the surfaces or membranes (if they be such) at which excitation occurs. The capacities, however, of biological membranes may be large and their insulating properties high, so that changes of impedance and of current distribution may be important, even at the relatively low frequencies we have used. It will be interesting to study excitation by alternating current in the much simpler systems provided by the non-medullated nerve of marine invertebrates. The possible effects of (d) are still more difficult to estimate. It might be practicable to avoid them altogether by examining the I - n relation in single nerve fibres.

The Effect of Frequency on the Threshold—The effect of high frequency as such was examined by Bugnard and Hill for maximal stimulation by short condenser discharges; they studied the variation of response with frequency. Their results are not directly applicable to the present investigation, where the response taken as index of excitation was constant, and as small as possible, and the variation of threshold with frequency was examined. It was necessary, therefore, to make special experiments to find whether, and to what extent, the threshold for alternating condenser discharges of very short duration varies with the frequency. A short duration was necessary since the maximum frequency desired was so high that, with the commutator used, not more than 200 μ sec. was available for each discharge, and it was thought best to keep the value of RC short compared with this and with the interval between successive shocks. The duration was so short that it lay almost in the region in which the "constant quantity" relation (Hill, 1936a, p. 317; 1936b, p. 442) obtains. Thus the experiments were directed to finding the effect of frequency as such with alternating condenser discharges of constant duration so short that they could be regarded as effectively "instantaneous".

The method employed was that described by Hill (1934, fig. 1), employing a commutator with 46 segments. This could be driven at any speed up to nearly 100 revolutions per second, and so could give two-way condenser discharges at any frequency n up to 2000 per second

(n discharges each way). This was used with capacity $C = 0.005 \mu\text{F.}$ and shunt resistance, $R_1 = 4000$ ohms. In series with the nerve was a resistance $R_2 = 50,000$ ohms, which together with the nerve resistance made about 100,000 ohms, and reduced the resultant discharge resistance R to about 3800 ohms. Thus RC was just less than $20 \mu\text{sec.}$ The resistance R_1 consisted chiefly (2800 ohms) of a very fine resistance wire wound non-inductively round the "hot" junctions of a thermopile connected to a Moll galvanometer.

The resistance R was purposely made rather high and the capacity C small, because at very high speeds the resistance at the contact of the brush with the commutator might not otherwise be negligible, and there was danger that variable discharges might occur. With $R = 200$ ohms and $C = 0.1 \mu\text{F.}$, for example, which give the same value of RC , this was very evident. That the arrangement ultimately used was not affected by this error was controlled, for every frequency used, by recording the reading of the galvanometer connected to the thermopile and verifying that this was proportional to the rate of energy liberation E^2Cn of the stimulus. If the resistance at the contacts on the commutator were an appreciable fraction of the discharge resistance R , a proportional amount of the energy would be lost at the contacts and the reading of the galvanometer would be less. Since this was not so, the resistance at the contacts was negligible compared with R and the discharges must have occurred sufficiently nearly from potential E and with time-constant RC . It is essential to control the strength and character of the condenser discharges in this way, for otherwise there is serious risk that at high frequency the threshold will appear to be raised owing to the stimuli being, in fact, less effective than they were intended to be.

In most experiments the index of constant excitation was a very small steady action current response (2 mm.) of the nerve, recorded by a sensitive galvanometer. The greatest response of the nerve to a maximal stimulus at the most effective frequency was 150–300 mm. In a few experiments, at the lower frequencies only, a minimal steady muscle contraction was used as index, for a special purpose referred to below. The index response was chosen as small as could be read accurately. A constant fraction of the maximal response at each frequency would have been better but this could not be used since, at high frequency, the maximal response requires a very strong stimulus and this affects the condition of the nerve and subsequent readings of the threshold. With a constant index, the error is least when the index itself is least.

The distant stimulating electrode lay on an injured part of the nerve, so that excitation was at one point only and of frequency n (not $2n$).

In every determination of the threshold the commutator was set running at a measured speed and then the potential E was adjusted to give the threshold response desired. The energy reading of the hot wire thermopile was next made, to control the stimulus. The speed of the commutator was then altered, E was determined again and the energy measured. Thus the threshold E was determined for a series of frequencies n .

The results were unexpected. At ordinary (rather high) temperatures (average 27° C.) the effect of frequency on the threshold was negligible, at least within the range 200–2000 double shocks per second. It was not advisable to drive the commutator much faster. The effective range, however, could be increased by cooling the nerve, and 12 experiments were made at about 7° C. Assuming a temperature coefficient $Q_{10} = 2$, a frequency of 2000 shocks per second at 7° C. is equivalent to one of 8000 at 27° C. At 7° C., the influence of frequency was rather variable, but the average effect of passing from 60 to 2000 double shocks per second was to raise the threshold by 23%. At 7° C., however, the maximal response was very much less at high frequencies than at low (*cf.* Bugnard and Hill, 1935*b*, *c*), and the small constant index, therefore, was a larger fraction of maximal. At higher frequencies the increase of response with increase of stimulus strength was rather slow (*cf.* experiment 2 of Table XVI), and we suspect that the effect of frequency on the threshold was really only apparent and due to taking too great an index response at higher frequencies. This could not be avoided without measuring the maximal response at each frequency and taking a constant fraction of it; which would probably have done more harm than good, by altering the condition of the nerve (owing to the strong stimulus necessary) and making the results unreliable. In any case the measurements with alternating current were made in the same way, with a small constant index response, so the two sets of experiments, with condenser discharges and with alternating current respectively, are strictly comparable.

In a low frequency range (45–200 per second) at the higher temperature there was a *fall* of threshold with increase of frequency. This was not large, but nevertheless quite definite, occurring in every one of 12 experiments and averaging 14%. It was not found at a low temperature; possibly it could have been detected in a still lower frequency range. The fall observed at the higher temperature was probably due largely to the fact that in this frequency range the maximal response is greater at the higher frequency, so that the small constant nerve response used as index was a larger fraction of maximal at the lower frequency and therefore required a stronger stimulus. That this was so was indicated

TABLE XVI—EFFECT OF FREQUENCY (n) ON THRESHOLD (E, VOLTS) OF TWO-WAY CONDENSER DISCHARGES OF SHORT DURATION (n DISCHARGES EACH WAY PER SECOND)

<i>Typical Experiments</i>												
(1) 8.5° C. Nerve response.												
n	137	240	322	479	1110	1900	1915	1005	323	185		
E	17.7	17.9	18.5	19.3	19.9	19.5	19.2	19.2	18.8	18.0		
(2) 8° C. Nerve response.												
n	58	228	315	544	830	1160	1960	845	327	70		
E	12.6	13.6	14.4	15.8	16.2	16.7	19	18	15.5	13		
At $n = 1960$, E, volts												
Response, min.			16	17	17.9	19.5						
			0.5	1.0	1.5	2.3						
(3) 27° C. Nerve response.												
n	30	60	212	309	654	1160	1940	680	328	60	40	
E	6.85	6.3	5.9	5.8	5.93	5.95	6.1	6.15	5.93	6.25	6.55	
(4) 28.5° C. Nerve response.												
n	50	188	321	560	845	1178	1975	572	72	53		
E	5.2	4.7	4.6	4.6	4.7	4.6	4.6	4.65	5.2	5.7		
(5) 25° C. Muscle response.												
n	25	39	68	122	171	372	122	26				
E	10.45	10.3	10.1	10.0	9.9	9.9	10.2	10.6				
(6) 22° C. Muscle response.												
n	20	37	63	116	210	167	118	67	38	23		
E	5.05	4.95	5.0	5.0	5.15	5.05	5.05	4.95	5.1	5.2		

SUMMARY

- (A) Low temperature, average 7° C., 12 experiments. Nerve response. Percentage rise of threshold E between $n = 60$ (average) and $n = 2000$: 10, 0, 27, 8, 55, 40, 0, 13, 20, 20, 45, 45; mean 23%.
- (B) High temperature, average 27° C., 12 experiments. Nerve response.
 (i) Percentage fall of threshold E between $n = 45$ (average) and $n = 150-250$: 13, 12, 28, 15, 10, 18, 18, 10, 5, 14, 9, 16; mean 14%.
- (ii) Percentage rise of threshold E between $n = 150-250$ and $n = 2000$: 3, 3, 10, 0, 0, 0, 0, -5, -7, 0, 3, 3; mean 1%.
- (C) High temperature, average 24° C., 12 experiments. Muscle response. Percentage fall of threshold E between $n = 25$ (average) and $n = 150$ (average): 5, 6, 5, 4, 2, 7, 10, 2, 3, 10, 3, 5; mean 5%.

by experiments made with minimal muscle response as index. The effect of frequency on the maximal muscle response is very small in this range (25–150 double shocks per second) so that the index taken was nearly a constant fraction of maximal. With muscle response as index the fall of threshold with increase of frequency, in this low frequency range, was very small, averaging only 5% in 12 experiments. This remaining difference is probably genuine, and of the same nature as the greater difference, described in § III above, between the constant current rheobase and the rheobase measured by repetitive pulses.

The experiments at higher frequencies could not be made with muscle response as index, owing to immediate relaxation of the muscle due to Wedensky inhibition.

We conclude, therefore, that the effect on the threshold of frequency of excitation as such, with alternating condenser discharges of constant short duration, is very small. In the low frequency range the threshold falls slightly as the frequency rises to about 200 double shocks per second. Above that, however, it remains practically constant. Any effect observed in the higher range is probably due to the fact that a constant index response, however small, represents a greater fraction of maximal response at high frequency than at low, and therefore requires a greater stimulus.

We found above (fig. 12) that with alternating current the I - n relation at low temperature tends to bend upwards at the higher frequencies. The experiments were made with small constant electric response of the nerve as index; they are comparable, therefore, with the present experiments made with condenser discharges. The bending upwards is probably due to the effect of frequency as such in raising the apparent threshold under these conditions. At high temperature (fig. 11) there was no regular tendency for the I - n relation to bend up at the higher frequencies; this corresponds to the absence here of a regular effect of frequency as such on the threshold determined with alternating condenser discharges.

The absence, at higher temperatures, of any regular effect of frequency on the threshold shows that the regular deviation of the I^2 - n^2 relation, for alternating current, from its theoretical linear form cannot be attributed to frequency as such. That deviation can scarcely be due to failure of the simple theory below a certain duration of the individual half-wave, since with condenser discharges the theoretical E-RC relation holds down to very low values of RC. If, for example, the deviation from the linear I^2 - n^2 relation begins at 650 cycles per second, this represents a duration of half-wave of 770 μ sec., and the E-RC relation has been verified down to about $RC = 20 \mu$ sec. (Hill, 1936*b*). We must probably look for the cause of the divergence from theory in the changes of current

distribution inside the nerve, or fibre, with alteration of frequency, due to electrical capacity or polarization as discussed above.

The possibility (*d*) was referred to above (p. 121) that a change of frequency might lead to a change in the current distribution inside a nerve, and so—for a minimal response—to the stimulation of different fibres at different frequencies. That some such random cause produces the varied curves of fig. 11 is suggested by the fact that a single nerve excited at different points at the higher frequencies may produce just such a variety of I - n curves. At the lower frequencies, however, the I^2 - n^2 relation was consistent (*a*) in being linear up to 500–1000 cycles per second, (*b*) in giving nearly the same value of k at different points of the nerve, and (*c*) in bending away from the linear relation at higher frequencies as in fig. 10*a*. In six experiments, in which separate I^2 - n^2 series were made at each of three points (the other electrode being in a common injured region) the three values of k lay within the following limits: 0.38–0.41; 0.35–0.36; 0.51–0.59; 0.35–0.48; 0.4–0.5; 0.37–0.47. At lower frequencies, therefore, a consistent relation is found from one point to another, but not at higher frequencies.

The absence of any important effect on the threshold, of frequency of excitation by condenser discharges of very short duration, contrasts, at first sight somewhat strangely, with the rather large effect of frequency, described by Bugnard and Hill (1935*b, c*), on the maximal response. Strong high frequency excitation appears to prolong the refractory state, even the impulses which are ineffective, owing to falling in the refractory periods of their predecessors, having an influence in slowing the return of normal excitability. This lengthening of the refractory period causes the nerve to respond less times per second and so diminishes the maximal response. With threshold excitation, however, the situation is different. After one effective stimulus the nerve will be absolutely refractory for a certain interval, then relatively refractory, but its normal excitability will ultimately return, and finally it will respond to the same strength of stimulus as before. Apparently the ineffective shocks which fall during the absolute and relative refractory periods do not prevent—though they may delay—the threshold from returning ultimately to its initial level. The experiments of Bugnard and Hill showed that the return to normal excitability is slowed (the refractory period lengthened) in high frequency excitation by strong shocks most of which fall within the refractory period. Thus the nerve does not respond so often and the maximal response is lessened. The present experiments, however, prove that even if the return to normal excitability is slowed by the weaker shocks used for determining the threshold, it does finally occur; high frequency as such

does not prevent the nerve from continuing to respond, though perhaps at longer intervals, to a given threshold shock. The apparent contrast, therefore, is resolved.

One consequence of the observed fall of threshold in the low frequency range, with rise of frequency as such, must be considered, viz., its influence on the I - n relation for alternating current in that region, and in particular its effect on the value of λ calculated from the I^2 - $1/n^2$ line below the optimum frequency. Fortunately, the change of threshold with frequency is small; indeed, when minimal muscle response is used as index of excitation almost negligible. The simplest way to test the effect is by trial with actual numbers. Taking, for example, any of the results of Table XIV, let us assume (for simplicity) a given fall of threshold uniformly occurring between 30 cycles per second and the optimum frequency; let us correct for this and plot corrected I^2 against $1/n^2$. The result will be (cf. figs. 3*b* and 9*b*) a line which rises rather less to the left and bends up rather more to the right, giving a value of λ which is slightly greater than originally calculated. The percentage correction in λ will not be more than the percentage change in the threshold over the range considered. This is of little significance. The argument of the preceding Sections of this paper will not be affected.

Since frequency as such has no effect on the threshold above about 200 cycles per second the value of k calculated from the I^2 - n^2 line requires no correction.

Divergence from the General Theory—It was disappointing at first, but it is scarcely a matter for wonder, that the divergences from the simple theory discussed in this Section should occur: what was astonishing rather was that the theory agreed with the facts so far and so well. We are inclined, indeed, to believe that in three respects at least the apparent agreement of observation with theory has been slightly improved by favourable chance: (1) the approximate equality of the "true" and the "observed" rheobase, due—it is suggested in § III above—to a balance between electrotonus and accommodation, has probably exaggerated the accuracy of fit of experimental observations to the theoretical voltage-capacity curve (Hill, 1936*b*); (ii) the effect of frequency as such on the apparent threshold, lowering it more near the optimum frequency for alternating current than at lower frequencies, has given a slightly better linear relation between I^2 and $1/n^2$, on the low side of the optimum, than was expected; (iii) the fact that threshold excitation with alternating current occurs (fig. 1) at the moment when the nerve is just unaccommodated has probably eliminated disturbing factors present in other methods of stimulation. It could scarcely be hoped, in any case, that a general

statement which takes no account of the very complex physico-chemical structure of nerve would give a complete description of all the facts. When clear-cut divergence from the theory occurs we may expect to be able to use it to obtain more specific knowledge of the mechanism involved. The facts which fit the general theory cannot provide this knowledge. Such divergences, therefore, are to be welcomed, not for the sake of disproving the general theory, which fits so many of the facts so well that it can scarcely be altogether wrong, but for the sake of elucidating the physico-chemical details of the heterogeneous excitatory mechanism of which the general theory takes no account.

XII—DISCUSSION

Physiological literature contains many references to the excitatory effects of alternating current. von Kries (1884*a*) discovered the existence of an optimum frequency, depending on the temperature, and since he published in the same year a paper (1884*b*) on excitation by slowly increasing current, he probably had in mind the relation between what is now called "accommodation" and the rise of threshold on the low side of the optimum frequency. von Kries also described an *Anfangszuckung* more readily observed at a higher frequency, which was probably due to the rapidly vanishing exponential term referred to in § X above, but which he connected with the *Anfangszuckung* described by Bernstein which may actually have been due to a Wedensky effect.

Nernst's theory of electric excitation was first (1899) applied to the case of alternating current; and von Zeynek (1899) in Nernst's laboratory determined the relation between threshold and frequency for the excitation of human sensory nerves. Nernst in a later paper (1908) discussed alternating current excitation in greater detail, and a vanishing exponential term, analogous to that referred to in § X above, occurred in his equations. Einthoven (1900) examined nerve excitation by alternating current of very high frequency and found enormous currents to be required; from 6.5×10^4 to 10^6 cycles per second the threshold rose faster than the frequency; near 10^6 cycles per second a current of about 10 milliamperes was necessary, enough to heat the nerve 65°C. , in one second, whereas with direct current only about 0.6 microampere was sufficient. He stated correctly, contrary to a prevailing error based on a false idea of the specific resistance of living tissue, that the high frequency current travelled throughout the body and not merely on the surface of the nerve.

Wertheim-Salomonsen (1905) employed a singing arc to produce oscillations from 2.5×10^3 to 2×10^6 cycles per second; he did not

examine the lower range of frequencies; in the higher range he found the threshold to increase more rapidly than the frequency. The singing arc, however, as Nernst pointed out (*see* Reiss, 1907), is unsuited to produce a pure oscillation of the kind required to test any theory. Asher (1923) found the threshold to rise in direct proportion to the frequency in the higher range from 10^4 to 3×10^5 cycles per second, but he gave few experimental details and reported that even with 3×10^6 cycles per second he could obtain smooth tetani from the muscle-nerve preparation of the frog. Some leak, rectification, or other disturbance must have occurred, for otherwise the nerve would have been coagulated by heat by the current necessary. Krüger (1928) worked with frequencies of 400 cycles per second upwards and concluded that the threshold rises faster than the square root of the frequency (Nernst's "Law"). Plotting E^2 against n^2 one obtains roughly a straight line for his lower frequencies, and can calculate a k of about 0.3 msec. which is of the right order of size.

Lullies (1929, 1930*b*) and Achelis (1930), working with low frequencies on frog's nerve, confirmed the existence of an optimum frequency, while Renquist and Koch (1930) also found it on man. Gildemeister (1930), in a mathematical discussion of Nernst's theory of excitation by alternating current, examined the rapidly decaying exponential term depending on initial phase (*cf.* § X above), and concluded that this would have the greatest effect when the current began at phase zero. Buchloh (1931) examined the influence of initial phase experimentally, and found a small effect at 50–100 cycles per second, but none at 200, 300, and 500 cycles: probably he could not go to high enough frequencies, on the high side of the optimum. Blair (1932) tested an equation similar to equation (6) above, deduced from his theory of excitation, by measurement of the threshold at frequencies between 300 and 40,000 cycles per second. It was necessary to take three or four different values of k , in the different frequency ranges, in order to obtain a fit; the values of k deduced from the lowest frequency range were of the right order of size. Coppée's experiments (1934*a, b, c*) have been fully discussed in various Sections above.

The experiments on frogs' nerves described in this paper fit the present theory very well within a range of frequencies ending usually at 400–1000 cycles per second, depending on the temperature. Above that range, other factors, discussed in § XI, are involved. The papers cited above dealt chiefly with the higher range, and had no "base-line" of a satisfactory theory, applicable to the lower range, from which they could start. Nernst's deduction that I should be proportional to $\sqrt{n}$ obviously does not fit the facts. Moreover, at least until recently, the experimental

technique has been difficult, and liable to various errors and disturbances. For a quantitative treatment of the problem most of the older results are of doubtful value. Qualitatively the more important facts were described by von Kries more than 50 years ago.

We doubt whether the experiments recorded hitherto, on stimulation of human nerves and sensory end organs, have much quantitative significance. The large electrical capacity of the skin, and the certainty that capacitative effects in the tissues will affect current distribution as frequency is altered, render deduction from human experiments very difficult. We have made a number of such experiments ourselves, using an amplifier with the oscillator described in § II, and measuring directly the actual current passing into the body with a rectifier and Moll galvanometer; readings were quite easily made and the thresholds were sharp, but the results fitted no particular theory and we could deduce nothing from them. It is not easy to see how changes in current distribution in the tissue, with changes of frequency, can be avoided when membranes of high resistance and considerable capacity lie in all directions in the path of the current. The impedance of the nerve sheath itself may be less at a higher frequency, the current traversing the tissue therefore may more readily flow through the nerve, and so the threshold tends to rise with frequency less rapidly than it should. The stimulation of sensory end organs in the skin might be a more hopeful method of research, except for the presence in their immediate neighbourhood of the large electrical capacity of the skin itself. There can be little security that the current takes the same path at different frequencies, and if not the apparent accuracy of such experiments is illusory. We had hoped that a stable experimental object, viz., the human nerve or sensory organ, would provide rather special opportunities of precise measurement. Actually we have been forced to conclude that the experiments made on frogs' nerves give much more useful information than any which we could obtain on man.

We are indebted to Mr. J. L. Parkinson for his continual skilled assistance. The sensitive galvanometers, and the photoelectric apparatus for making alternating current of low frequency, were constructed by Mr. A. C. Downing.

Expenses were met by the Foulerton Fund of the Royal Society.

SUMMARY

The theory of nerve excitation by alternating current is discussed. The relation between I and $\log n$ should be a symmetrical curve with vertical axis and vertex at the optimum frequency. The optimum frequency

depends on the product $k\lambda$, the shape of the I - $\log n$ curve on the ratio k/λ , where k and λ are the time constants of excitation and accommodation respectively.

The relation between I and n can be considered in two regions, (i) below, and (ii) above the optimum frequency. In (i) there should be a nearly linear relation between I^2 and $1/n^2$, from the slope of which λ can be determined. In (ii) there should be a nearly linear relation between I^2 and n^2 , from which k can be calculated.

Experimental methods are described in detail. The agreement of the theoretical I - n curve with observation is good, and the values of k and λ found with alternating current agree closely with those determined by other methods.

The "true" rheobase deduced from alternating current measurements is, on the average, precisely the same as the rheobase observed with repetitive constant current pulses of sufficient duration. The agreement is probably due to a balance between accommodation and electrotonus.

When the inter-electrode distance is reduced, k is diminished. The effect is the same whether k is obtained by condenser discharges or by alternating current. A reduction of inter-electrode distance has little, if any, effect on λ .

The optimum frequency increases, with a Q_{10} of about 2.5, when the temperature rises. Experimental values agree with those calculated from k and λ .

The value of λ/k is not constant. It varies widely from one nerve to another, it depends upon the Ca-ion concentration, the inter-electrode distance and probably on the temperature. In the present investigation, values from 7.3 to 45 have been observed. By depriving the nerve of calcium far higher values can be obtained, but these are not suited to measurement by alternating current.

The theory predicts the possibility of a single initial excitation by a current below the steady threshold, depending on the phase at "make". This has been verified experimentally, both above and below the optimum frequency.

The effect of frequency as such on the threshold has been investigated with extremely short condenser discharges of constant form but of variable frequency. It is very small. The apparent contrast of this result with the rather large effect of frequency on the maximal response is discussed.

The relation observed between I and n with alternating current usually begins to deviate from that predicted by the theory at a frequency of 400–1000 cycles per second, depending on the temperature. This deviation

is not due to the effect of frequency as such, nor can it be caused by failure of the theory to deal with currents of durations corresponding to the frequencies in question. The causes of the deviation are discussed.

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Further Observations on the Oestrogenic Activity of Synthetic Polycyclic Compounds

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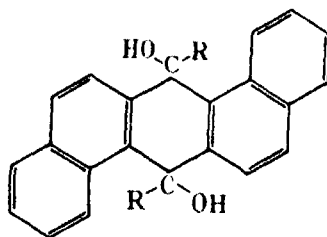
(Communicated by E. L. Kennaway, F.R.S.—Received 6 May, 1936)

In a former communication (Cook, Dodds, Hewett, and Lawson, 1934) we recorded the oestrogenic activity of a number of polycyclic aromatic and hydroaromatic compounds. The most active substances were found among a series of diols related to the carcinogenic hydrocarbon, 1:2:5:6-dibenzanthracene. We have now extended our investigation to seven new members of this series and have also examined a variety of diols of analogous structure, but containing other ring systems.

Table I summarizes the results obtained in tests for oestrogenic activity carried out with 9:10-dihydroxy-9:10-dialkyl-9:10-dihydro-1:2:5:6-dibenzanthracenes. In none of the new compounds has the activity exceeded that of the most potent compound previously described, namely, the di-*n*-propyl derivative in this series. Among the straight-chain dialkyl compounds this di-*n*-propyl compound represents a peak of activity in a series of which the first (methyl) and higher (*n*-amyl, *n*-hexyl) members are inactive. Two branched-chain compounds (*iso*-propyl, *iso*-butyl) are fairly highly active, but show diminished activity by comparison with the corresponding straight-chain isomerides. This is particularly marked for the *iso*-propyl compound, which is about ten times less active than the *n*-propyl compound. In view of the high order of activity of the *n*-propyl compound, it is remarkable that the allyl

compound has given negative results when injected into ovariectomized rats in 10 mg. doses. The benzyl group, which in structure and chemical activation bears some resemblance to the allyl group, has not the inhibitory effect of the allyl group on the oestrogenic properties of the

TABLE 1—DERIVATIVES OF 9:10-DIHYDROXY-9:10-DIHYDRO-1:2:5:6-DIBENZANTHRACENE



Substituent group (R)	No. of rats	Dose in mg.	% positive
Methyl	5	100	Nil
Ethyl	5	1.0	100
<i>n</i> -Propyl	15	0.025	40
<i>iso</i> -Propyl	10	0.5	100
"	10	0.25	70
Allyl	3	100	Nil
<i>n</i> -Butyl	14	0.1	50
<i>iso</i> -Butyl	5	1.0	80
<i>n</i> -Amyl	5	100	Nil
<i>cyclo</i> -Pentyl	5	1.0	100
"	5	0.5	60
<i>n</i> -Hexyl	3	100	Nil
<i>cyclo</i> -Hexyl	5	10	Nil
Phenyl	5	10	Nil
Benzyl (Cook, 1931)	5	10	60
"	5	1.0	Nil

molecule, for the benzyl compound was active in 10 mg. doses. Another point of interest lies in the effect of cyclization of the aliphatic chain in some of these compounds. For the activity of the molecule which was lost by lengthening the chain to *n*-amyl was restored in the *cyclo*-pentyl compound, which gave positive results in 0.5 mg. doses, and thus had approximately the same order of activity as the *iso*-propyl and *n*-butyl compounds. At first sight it might appear that this effect supports the view that oestrogenic activity is associated with the linear dimensions of the molecule, for cyclization of the *n*-amyl chains will tend to reduce the breadth of the molecule to that of the lower (active) members of the series. That this is a determining factor is rendered unlikely, however, by the negative results obtained with the *cyclo*-hexyl and phenyl compounds.

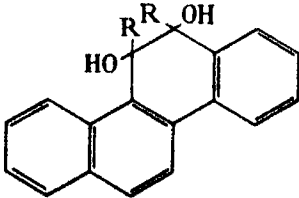
These considerations illustrate the difficulty of formulating any general correlation with molecular structure of this type of biological activity.

In any case it would be unwise to generalize at the present stage, as no information is available concerning the configuration of the molecule in these compounds. The comparative inaccessibility of 1:2:5:6-dibenz-anthraquinone, and the poor yields of diols obtained in many of the Grignard condensations have precluded the preparation of sufficiently large quantities of the crude diols to attempt the separation of *cis* and *trans* forms in order to study the influence of configuration on the oestrogenic activity. In the absence of such pairs of stereo-isomerides we have been unable to determine whether our compounds belong to the *cis* or to the *trans* series. Moreover, we are by no means prepared to assert that the diols which we have examined all belong to the same stereochemical series, and in connexion with other biological properties it is well known that slight configurational changes may be accompanied by a profound change in biological activity.

In view of the slight pro-oestrous activity of a keto-octahydroanthracene, reported in our former communication, it seemed of interest to examine a series of homologues of 9:10-dihydroxy-9:10-dihydroanthracene. Seven compounds of this type have been prepared and tested. These are tabulated in a later section. None of these compounds has shown any oestrogenic activity; six of them were injected into rats in 100 mg. doses, and one of them (the *cyclo*-pentyl compound) in 10 mg. doses.

On account of the similarity of the chrysene ring system to that present in the natural hormones, we have also examined some diols related to chrysene. The results are recorded in Table II, which shows that although

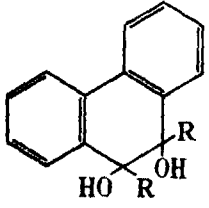
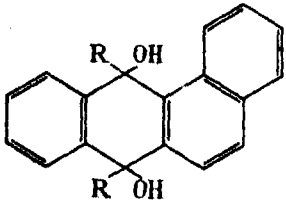
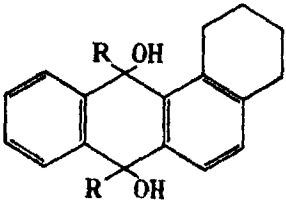
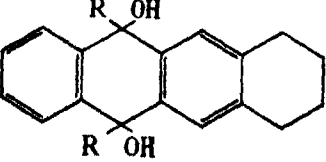
TABLE II—DERIVATIVES OF 1:2-DIHYDROXY-1:2-DIHYDROCHRYSENE

				
Substituent group (R)	Method of preparation	No. of rats	Dose in mg.	% positive
<i>n</i> -Propyl	—	5	100	Nil
<i>n</i> -Amyl	—	5	100	Nil
Phenyl	Cook and Galley (1931)	4	50	100
"	—	5	10	60
"	—	5	1.0	Nil
Benzyl	Cook and Galley (1931)	5	100	Nil

1:2-dihydroxy-1:2-di-*n*-propyl- and *n*-amyl- 1:2-dihydrochrysene were inactive, positive results were obtained with the corresponding diphenyl compound in 10 mg. doses. This indicates that the negative results obtained with the diphenyl compound in the dibenzanthracene series (Table I) is not due to any specific inactivating influence of the phenyl group.

Other diols of analogous structure have been prepared by the action of Grignard reagents on phenanthraquinone, 1:2-benzanthraquinone, and tetrahydro derivatives of 1:2- and 2:3-benzanthraquinones. These are enumerated in Table III which shows that oestrogenic activity was shown

TABLE III—DERIVATIVES OF PHENANTHRENE, 1:2-BENZANTHRACENE, AND 2:3-BENZANTHRACENE

Formula	Substituent group (R)	Method of preparation	No. of rats	Dose in mg.	% positive
	<i>n</i> -Propyl	Zincke and Tropp (1908)	5	100	Nil
	Phenyl	Werner and Grob (1904)	5	100	Nil
	α -Naphthyl	—	5	100	Nil
	Phenyl	Clar (1930)	4	100	50
	Phenyl	—	5	100	Nil
	<i>n</i> -Butyl	—	5	100	Nil
	Phenyl	—	5	100	Nil

by only one of these, namely, 9:10-dihydroxy-9:10-diphenyl-9:10-dihydro-1:2-benzanthracene; this produced oestrus in two rats out of four when injected in 100 mg. doses. The inactivity of the analogous compound in the dibenzanthracene series cannot be contrasted with these positive results, as the latter compound could only be administered in 10 mg. doses on account of lack of material. Negative results were obtained when pyrenic acid, an oxidation product of pyrene (Bamberger and Philip, 1887), was injected in the form of its water-soluble sodium salt in 100 mg. doses into five rats.

The statement of Butenandt and Störmer (1932) that the methyl ether of oestrone has no oestrogenic activity was made at a time when the comparative lack of specificity of this biological action was unappreciated, and it seemed of interest to determine to what extent methylation of the hydroxyl group depresses the activity. Negative results were obtained when doses of 10 γ of oestrone methyl ether were injected into five rats, but complete oestrus was obtained in all five rats following injection of doses of 50 γ . The activity of the methyl ether is thus between 10 and 50 times smaller than that of oestrone.

The biological activity of oestrone and androsterone is considerably enhanced by reduction of the 17-keto group to hydroxyl, a fact which suggested the examination for oestrogenic action of 1-hydroxy-1:2:3:4-tetrahydrophenanthrene. This was obtained by catalytic hydrogenation of 1-keto-1:2:3:4-tetrahydrophenanthrene, which we had previously found active in rats in 50 mg. and 100 mg. doses. The hydroxy compound was injected in 100 mg. doses into five rats, with negative results. In this case therefore, the effect of reduction of the carbonyl group resembles that observed with progesterone, the biological activity of which is destroyed when one of its keto groups is reduced (Butenandt and Schmidt, 1934). In this case, as in our case, the inhibition of activity cannot be ascribed to an abnormally high degree of specificity, for Klein and Parkes (1936) have recently shown that a certain degree of progestational activity is shown by a male hormone derivative which differs structurally from progesterone to an appreciably greater extent than does its reduction product.

In our earlier communication we reported that the synthetic oestrogenic compounds had the same biological action as the natural hormone, not only in producing vaginal changes in rats and mice but also in stimulating uterine enlargement, and in inducing premature puberty in immature animals. The observation was also made (Cook, Dodds, and Greenwood, 1934) that these compounds, like oestrone, could produce plumage changes in capons. We have now made the additional observation that

9:10-dihydroxy-9:10-di-*n*-propyl-9:10-dihydro-1:2:5:6-dibenzanthracene can replace oestrone in preparing the uterus for the endometrial proliferation brought about by progesterone. Moreover, Burrows (1935-36) has found that the pathological effects of continued administration of oestrogenic hormones may also be reproduced by this same compound. Thus, as far as we know there is no biological action of the naturally occurring oestrone which cannot be reproduced by the synthetic oestrogenic compounds which we have investigated.

TECHNIQUE

In the tests for oestrogenic activity, Wistar rats were employed throughout, and the compounds were injected in solution or suspension in sesame oil. Details of the number of rats used, the total quantity of substance administered, and the results obtained, are given in Tables I to III. In all cases we have adhered to the concentrations, and spacing of doses described in our earlier communication to which reference should be made for further information concerning the biological testing of the compounds.

PREPARATION OF MATERIALS

The diols which have been examined for oestrogenic action were all prepared by the action of Grignard reagents on the appropriate quinones, and, except where otherwise stated, we have followed the standard conditions already described (Cook, Dodds, Hewett, and Lawson, 1934). In the case of known compounds the properties agreed with those described in the literature, references to which are given in Tables I, II, and III. The properties of new compounds will now be described.

A—9:10-Dihydroxy-9:10-Dialkyl (or aryl)-9:10-Dihydro-1:2:5:6-Dibenzanthracenes

The *iso-propyl compound* formed colourless needles m.p. 288–290° (from benzene), and gave an intense blue solution in concentrated sulphuric acid. (Found: C, 84·8; H, 7·1. $C_{28}H_{28}O_2$ requires C, 84·8; H, 7·1%.)

The *allyl compound*, obtained by the use of allylmagnesium bromide (Gilman and McGlumphy, 1928), formed colourless prisms, m.p. 210–211° (from benzene), and gave a cherry-red colour with sulphuric acid. (Found: C, 85·9; H, 6·3. $C_{28}H_{24}O_2$ requires C, 85·7; H, 6·2%.)

The *iso-butyl compound*, m.p. 212° (from benzene-light petroleum), gave an intense blue colour with sulphuric acid. (Found: C, 84.8; H, 7.5. $C_{30}H_{32}O_2$ requires C, 84.85; H, 7.6%.)

The *cyclo-pentyl compound*, prepared using 10 molecular proportions of *cyclo-pentylmagnesium bromide*, formed colourless needles, m.p. $281-282^{\circ}$ (from benzene), and gave an intense blue colour with sulphuric acid. (Found: C, 85.8; H, 7.3. $C_{32}H_{32}O_2$ requires C, 85.7; H, 7.2%.)

The *cyclo-hexyl compound* (10 mols. of Grignard reagent) formed pale yellow needles, m.p. $279-280^{\circ}$ (from benzene), and gave a red colour with sulphuric acid. (Found: C, 85.9; H, 7.3. $C_{34}H_{36}O_2$ requires C, 85.7; H, 7.6%.)

The *phenyl compound* (10 mols. of Grignard reagent), crystallized from xylene in pale yellow prisms, m.p. 346° . (Found: C, 87.9; H, 5.6. $C_{34}H_{24}O_2$ requires C, 87.9; H, 5.2%.) This diol, which gave a purple colour with sulphuric acid, crystallized from ethyl acetate with solvent of crystallization. (Found, after air drying: C, 78.4; H, 6.4; loss when dried in a vacuum at 98° , 27.3%. $C_{34}H_{24}O_2$, $2C_4H_8O_2$ requires C, 78.7; H, 6.3; $2C_4H_8O_2$, 27.5%.)

B—9: 10-Dihydroxy-9: 10-Dialkyl (or aryl)-9: 10-Dihydroanthracenes.

With the exception of the allyl compound, which gave an olive green colour, these diols all gave red solutions in sulphuric acid.

The *allyl compound*, prepared with allylmagnesium bromide (Gilman and McGlumphy, 1928), formed colourless needles, m.p. $111-112^{\circ}$, from benzene-light petroleum. (Found: C, 81.8; H, 6.95. $C_{20}H_{20}O_2$ requires C, 82.15; H, 6.9%.)

The *n-propyl compound* formed colourless needles (from benzene-light petroleum), m.p. $171-172^{\circ}$. (Found: C, 80.8; H, 8.2. $C_{20}H_{24}O_2$ requires C, 81.0; H, 8.2%.)

The *n-butyl compound* formed colourless needles (from benzene), m.p. 158° . (Found: C, 81.5; H, 8.7. $C_{22}H_{28}O_2$ requires C, 81.4; H, 8.7%.)

The *n-amyl compound* formed colourless octahedra (from benzene), m.p. $179-180^{\circ}$. (Found: C, 81.7; H, 9.0. $C_{24}H_{32}O_2$ requires C, 81.75; H, 9.15%.)

The *cyclo-pentyl compound* formed colourless needles (from benzene), m.p. 216° . (Found: C, 82.7; H, 8.2. $C_{24}H_{28}O_2$ requires C, 82.7; H, 8.1%.)

The *phenyl compound* (Ingold and Marshall, 1926) and the α -*naphthyl compound* (Guyot and Stachling, 1905) of this series were also prepared and tested.

C—*Derivatives of Phenanthrene, Chrysene, 1:2-Benzanthracene, and 2:3-Benzanthracene.*

9:10-Dihydroxy-9:10-di- α -naphthyl-9:10-dihydrophenanthrene formed colourless needles (from benzene), m.p. 265–266°. (Found: C, 87.7; H, 5.3. $C_{34}H_{24}O_2$ requires C, 87.9; H, 5.2%.)

1:2-Dihydroxy-1:2-di-n-propyl-1:2-dihydrochrysene formed colourless needles (from benzene-light petroleum), m.p. 145°. (Found: C, 82.9, H, 7.5. $C_{24}H_{26}O_2$ requires C, 83.2; H, 7.6%.)

1:2-Dihydroxy-1:2-di-n-amyl-1:2-dihydrochrysene formed colourless needles (from benzene-light petroleum), m.p. 107–108°. (Found: C, 83.5; H, 8.5. $C_{28}H_{34}O_2$ requires C, 83.5; H, 8.5%.)

9:10-Dihydroxy-9:10-diphenyl-9:10:1':2':3':4'-hexahydro-1:2-benzanthracene, prepared from tetrahydro-1:2-benzanthraquinone (Schroeter, 1921) formed colourless needles (from benzene), m.p. 230–231°. (Found: C, 86.2; H, 6.3. $C_{30}H_{26}O_2$ requires C, 86.1; H, 6.3%.)

9:10-Dihydroxy-9:10-di-n-butyl-9:10:1':2':3':4'-hexahydro-2:3-benzanthracene, prepared from tetrahydro-2:3-benzanthraquinone (Schroeter, 1921), formed colourless octahedra (from benzene-light petroleum), m.p. 154°. (Found: C, 82.4; H, 9.0. $C_{26}H_{34}O_2$ requires C, 82.5; H, 9.1%.)

9:10-Dihydroxy-9:10-diphenyl-9:10:1':2':3':4'-hexahydro-2:3-benzanthracene formed colourless needles (from benzene-light petroleum), m.p. 222–223°. (Found: C, 86.4; H, 6.4. $C_{30}H_{26}O_2$ requires C, 86.1; H, 6.3%.)

D—1-Hydroxy-1:2:3:4-Tetrahydrophenanthrene

A solution of 1-keto-1:2:3:4-tetrahydrophenanthrene (10 gm.) in alcohol (500 cc.) was shaken with hydrogen and Adams's platinum oxide catalyst (1 gm.). The hydrogen absorption corresponded with addition of one molecule. After removal of the alcohol from the filtered solution, the residue was recrystallized from ligroin, and gave 1-hydroxy-1:2:3:4-tetrahydrophenanthrene (8.8 gm.), which formed colourless needles, m.p. 100–101°. (Found: C, 85.0; H, 7.3. $C_{14}H_{14}O$ requires C, 84.8; H, 7.1%.)

For characterization, the acetate was prepared by means of pyridine-acetic anhydride at 100°. It crystallized from hexane in colourless leaflets, m.p. 63–64°. (Found: C, 79.8; H, 6.7. $C_{16}H_{16}O_2$ requires C, 80.0; H, 6.7%.)

When the above carbinol was further hydrogenated with Adams's platinum catalyst in acetic acid solution at 4 atmospheres pressure, the hydroxyl group was lost, and the product was *s*-octahydrophenanthrene

(Found: C, 90.3; H, 10.1. Calc. for $C_{14}H_{18}$, C, 90.25, H, 9.75%.) The constitution of this hydrocarbon was established by oxidation with dilute nitric acid at 170° to mellophanic acid, isolated as its tetramethyl ester, m.p. 128°. (Found: C, 54.1; H, 4.5. Calc., C, 54.2; H, 4.55%.)

SUMMARY

In continuation of previous work (Cook, Dodds, Hewett, and Lawson, 1934), tests for oestrogenic activity have been carried out with some 18 diols prepared by the action of Grignard reagents on polycyclic quinones. Many of these are new compounds. The high activity recorded already for 9:10-dihydroxy-9:10-di-*n*-propyl-9:10-dihydro-1:2:5:6-dibenzanthracene has not been surpassed with any other synthetic compound. In the dibenzanthracene series of diols the effects of branching the chain of the alkyl group and of cyclization of the chain have been studied, and the work has been extended to analogous compounds containing the ring systems of anthracene, 1:2-benzanthracene, phenanthrene, and chrysene. In these new series oestrogenic activity of a low order was shown by 1:2-dihydroxy-1:2-diphenyl-1:2-dihydrochrysene and 9:10-dihydroxy-9:10-diphenyl-9:10-dihydro-1:2-benzanthracene. All other members of these four series which were examined were inactive.

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The Absolute Activity of Choline-Esterase

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The present investigation has been rendered possible by the results obtained in two separate, and at first apparently disconnected, fields of enquiry. One of us (Stedman, 1926), in a study of the relationship between chemical constitution and physiological action, had ascertained the molecular arrangement which was responsible for the characteristic pharmacological activity of physostigmine and, partly in conjunction with collaborators (1929–1931), had used this discovery to synthesize and investigate a number of compounds possessing physiological activities similar to that of physostigmine. During the same period, Loewi and Navratil (1926), in pursuing the well-known work of the former author on the humoral transmission of nerve action, discovered that small but pharmacologically active amounts of acetyl choline, as well as of the so-called vagus substance, underwent enzymic decomposition when treated with extracts of certain tissue, and that this decomposition was inhibited by the addition to such extracts of physostigmine, which discoveries were subsequently confirmed and extended by Engelhart and Loewi (1930), Matthes (1930), and other investigators. It thus became evident that the synthetic drugs should share with physostigmine the property of preventing the destruction of acetyl choline by blood and tissue extracts, provided this property was responsible, wholly or in part, for the physiological activity of the alkaloid. Another problem was, however, involved. No evidence was available as to the identity of the enzyme concerned in the process. It appeared probable that the destruction of acetyl choline was brought about by a simple esterase such as is known to exist in the liver, in blood serum, and in other tissues; nevertheless, the possibility remained that the effect was due to a specific enzyme. Stedman and Stedman (1931*b*, 1932) attempted to solve this problem indirectly by examining the influence of their synthetic drugs upon various ester-hydrolysing enzymes, and were able to demonstrate that, while these substances exerted a marked inhibitory action on liver and serum esterases, they were without such influence on other ester-hydrolysing enzymes such

as pancreatic lipase and kidney phosphatase. The drugs appeared to exert a specific inhibitory effect on simple esterases, and to this extent the experiments supported the view that these simple esterases were responsible for the destruction of acetyl choline originally observed by Loewi and Navratil. When, however, Stedman, Stedman, and Easson (1932) developed a technique for following the hydrolysis of acetyl choline by chemical methods, it was found that liver esterase was entirely without action on this ester, although the presence in horse serum of an enzyme which brought about its hydrolysis could be readily demonstrated. These and other results, in particular the purification of the enzyme, clearly proved that the hydrolysis of acetyl choline was brought about by a specific agent. To this new enzyme, which also catalyzes the hydrolysis of butyryl choline, the above-mentioned authors gave the name of choline-esterase.

From the foregoing account of the relationship between Loewi's work and that of the present authors, it will be clear that physostigmine and the analogous synthetic drugs will not only inhibit liver esterase but will also exert a similar action on choline-esterase. Experimental verification of this was, in fact, made in this laboratory shortly after the discovery of the specific nature of choline-esterase, but publication was postponed until the qualitative results could be supplemented by quantitative ones. When such a detailed examination of the drugs was commenced, their inhibitory activity towards choline-esterase was found to be much greater than towards liver esterase. The amount of inhibitor required to produce a large inhibition of the enzyme proved, in fact, to be so minute that there appeared to be a possibility of utilizing this property of the drugs for determining the absolute rate of hydrolysis of a choline ester by choline-esterase, a possibility which has been developed in the present investigation.

As a basis for this work the assumptions were made that the drugs produce their inhibitory action by combining with the enzyme, that the mechanism of the combination is identical with that concerned in the combination between enzyme and substrate, and that consequently one molecule of drug will inhibit the activity of one unit or active centre of enzyme by preventing the combination between the latter and its substrate. In support of these assumptions is the fact that all the inhibitors examined resemble acetyl choline structurally in containing an ester as well as a basic grouping, and it is, presumably, through the intervention of these molecular arrangements, common to both substrate and inhibitor, that combination with the enzyme takes place. The inhibitors, however, since they are not decomposed at an appreciable rate by the enzyme,

remain attached to the active group of the latter and hence prevent the combination between it and its substrate.

If these assumptions be accepted, it is clear that the "molecular concentration" of the enzyme and hence the absolute rate of hydrolysis of the substrate, *i.e.*, the number of molecules of substrate hydrolysed by one active centre of enzyme in one second, could be readily calculated from the results of a determination of the inhibition produced by a known concentration of inhibitor, provided a method were available for ascertaining the amount of the inhibitor actually combined with the enzyme. For this purpose it has been assumed that the reaction between the enzyme and inhibitor takes place according to the equation $E + I \rightleftharpoons EI$ and is governed by the law of mass action. If $[E]$, $[I]$, and $[EI]$ represent respectively the original concentrations of enzyme and inhibitor and the concentration of the compound formed by interaction between the two

$$\frac{([E] - [EI])([I] - [EI])}{[EI]} = K. \quad (1)$$

Now the fractional inhibition i produced by the inhibitor, a magnitude which can be determined experimentally, is numerically equal to $[EI]/[E]$. Substituting $[EI] = i[E]$ in (1) and rearranging, we find

$$[I] = i \left(\frac{K}{1-i} + [E] \right) \quad (2)$$

or

$$\frac{[I]}{i} = \frac{K}{1-i} + [E]. \quad (3)$$

By determining the inhibition produced by various concentrations of inhibitor, the concentration of enzyme being maintained constant, and by plotting the values for $[I]/i$ so obtained against $1/(1-i)$, it is clear from equation (3) that, provided the various assumptions made are correct, a straight line will result, the intercept of which on the axis of $[I]/i$ will give the value of $[E]$ and the slope that of K . From the value of $[E]$ thus obtained the number of molecules of substrate hydrolysed in a given time by one active centre of enzyme can be readily ascertained experimentally.

The above application of the law of mass action has involved the assumptions (1) that the reaction between enzyme and inhibitor is a reversible one, (2) that the compound enzyme-inhibitor is entirely without enzymic activity, (3) that the inhibition is apparently non-competitive, *i.e.*, that the inhibitor is not displaced by the substrate from its compound with the enzyme to an extent which appreciably affects the experimental

determination of the inhibition, and (4) that the inhibitor is not decomposed by the enzyme. Experimental verification of the first three of these assumptions is recorded below. The fourth assumption, however, has proved to be incorrect. Even with the most stable of the inhibitors examined, an amount of decomposition, as shown by the return of the activity of the enzyme, sufficient to impair the accuracy of the results occurs during the experimental period. The rate of this decomposition is exceedingly small and would be negligible were it not for the necessity, no doubt due to the minute concentration of inhibitor employed, of leaving the latter in contact with the enzyme for at least nine hours before measuring the inhibition, in order to ensure that equilibrium between the two shall be attained. It is possible to apply an empirical correction for this decomposition of the inhibitor, but it has been deemed preferable to use the following more accurate method.

It is shown below that the particular inhibiting urethane chosen for this work is stable in aqueous solution but is slowly decomposed by choline-esterase. The rate of this decomposition is assumed to be proportional to the concentration of enzyme-inhibitor compound and hence to the inhibition, *i.e.*,

$$-\frac{d[I]}{dt} = ki, \quad \text{or} \quad \frac{d[I]}{di} \cdot \frac{di}{dt} = -ki. \quad (4)$$

But, by differentiating [I] with respect to *i* in equation (2),

$$\frac{d[I]}{di} = \frac{K}{(1-i)^2} + [E].$$

Substituting this value of $d[I]/di$ in equation (4) and integrating the resulting expression,

$$\frac{-k}{[E]} \{t\}_{i_1}^{i_2} = \left\{ \left(\frac{K}{[E]} + 1 \right) \log i + \frac{K}{[E]} \left(\frac{1}{1-i} \right) - \frac{K}{[E]} \log(1-i) \right\}_{i_1}^{i_2} \quad (5)$$

or

$$\log \frac{i_2}{i_1} = \frac{K}{[E]} \left\{ \log \frac{i_1(1-i_2)}{i_2(1-i_1)} + \frac{i_1-i_2}{(1-i_1)(1-i_2)} \right\} + \frac{-k}{[E]} (t_2 - t_1). \quad (6)$$

If $t_2 - t_1$ is constant, equation (6) is linear with respect to

$$\log \frac{i_2}{i_1} \quad \text{and} \quad \left\{ \log \frac{i_1(1-i_2)}{i_2(1-i_1)} + \frac{i_1-i_2}{(1-i_1)(1-i_2)} \right\}.$$

Hence, from a series of determinations of i_1 and i_2 in which the interval $t_2 - t_1$ is maintained constant, numerical values of $K/[E]$ and $-k/[E]$

can be obtained as slope and intercept respectively of the straight line which results from plotting these two expressions.

If, now, $i_1 = 0.95$ is selected as the maximum value to be dealt with, a curve connecting t and i can be constructed from equation (5) by inserting the limits $(0, 0.95)$, (t, i) . This curve can then be employed, as has been done on p. 157 to correct observed inhibitions for the decomposition of the inhibitor. With the corrected values so obtained, the "molecular concentration" of the enzyme can be determined graphically by means of equation (3).

EXPERIMENTAL METHODS

Preparations of choline-esterase were made from blood-serum from the horse by the method described by Stedman and Stedman (1935). For the determination of the activity of such preparations the titration method of Stedman, Stedman, and Easson (1932), as modified by Stedman, Stedman, and White (1933), was used in the following form. To 100 cc. of water maintained at 30°C . in a thermostat were added 1 cc. of a molar solution of the substrate, 5 drops of an aqueous solution of indicator in the form of its sodium salt, and a suitable volume of enzyme or enzyme-inhibitor mixture. The solution was then brought to the desired p_H , as shown by comparison of the colour of the solution with a standard, by the addition of 0.02 N NaOH and maintained at this acidity by the addition, drop by drop as required, of further quantities of the alkali. Burette readings were taken every five minutes and the experiment continued for a total period of twenty minutes. Under the conditions chosen, the amount of hydrolysis was invariably proportional to the time; the individual five-minute readings are not therefore quoted in the various results given below. Bromothymol blue was used as indicator for the experiments carried out at p_H 6.8 and 7.4, while cresol red was employed in the few experiments made at p_H 8.0.

REVERSIBILITY OF INHIBITION

On dialysing a mixture of eserine and blood for 24 hours, Matthes (1930) observed a complete return of enzymic activity towards acetyl choline and concluded that the inhibitory process was a reversible one. In view, however, of our discovery that urethanes of the eserine type are slowly decomposed by choline-esterase, these experiments can no longer be regarded as a rigorous proof of reversibility in the sense demanded by our investigation. We are, in fact, of the opinion that the return of enzymic activity observed by Matthes was largely due to the combined

effect of the removal by dialysis of the free eserine present in the mixture and the destruction by the enzyme itself of that in combination. We have therefore reinvestigated this problem, using for this purpose purified solutions of choline-esterase and an inhibitor, prostigmine, which is decomposed by the enzyme much more slowly than is eserine. Nevertheless, a direct demonstration of the reversible nature of the combination between enzyme and inhibitor has proved to be a matter of considerable difficulty. Three factors complicate the apparent simplicity of the process of dialysis for this purpose. The first is the extraordinary affinity of the inhibitor for the enzyme, as a result of which a concentration of the former of the order of 10^{-9} molar suffices to produce a considerable inhibition. If, as has been assumed, the reaction is a reversible one and is represented by the equation $E + I \rightleftharpoons EI$, this great affinity must clearly mean that the rate of the reverse reaction is exceedingly slow relatively to the direct one. Since, moreover, a period of some nine hours must elapse after mixing enzyme and inhibitor before equilibrium is attained, it is evident that a much longer period will be necessary to remove by dialysis the inhibitor which is in combination with the enzyme. This difficulty could be readily obviated by prolonging the period of dialysis, were it not for the remaining two factors, namely, the decomposition of the inhibitor by the enzyme, and the instability of the enzyme itself when submitted to prolonged dialysis.

As a result of these complications it has not been possible to prove the reversibility of the process by the direct method of dialysis. Some indirect evidence of a fairly conclusive nature has, however, been obtained. Thus, it has been possible to show by two methods that part of the inhibitor employed in a particular experiment is not in combination with the enzyme but remains in the dispersion medium. In one of these methods a mixture of enzyme and inhibitor has been allowed to reach equilibrium, when a further quantity of enzyme has been added to the mixture. Inhibition of the added enzyme was then observed to occur in the normal manner. The following typical experiment will illustrate this point.

An enzyme preparation was made by mixing 2 cc. of the original solution of choline-esterase with 0.5 cc. of a bicarbonate-carbon dioxide buffer of p_H 7.4 and sufficient water to bring the total volume to 21 cc. To 10 cc. of this preparation were added 2 cc. of a 2×10^{-7} molar solution of prostigmine, this particular inhibitor being chosen for reasons which will be discussed below. At the same time a control solution was prepared by mixing 10 cc. of the same enzyme preparation with 2 cc. of water. After an interval of 24 hours the activity of 1 cc. of each of these

two solutions towards butyryl choline was determined in the usual manner, the results being: control, 5.20 cc.; inhibited solution, 0.75 cc.; whence $i = 0.85$. To 2 cc. of each of these solutions was now added 0.1 cc. of the original solution of choline-esterase, giving solutions C_1 and P_1 respectively, suitable control solutions, designated as C_2 and P_2 , being made by the addition of water in place of enzyme. The activities of these solutions, using 0.5 cc. in each case, were then determined with the following results:

Time after mixing, hours	C_1	P_1	C_2	P_2	i
0	3.95	1.90	—	—	0.52
24	3.85	1.30	—	—	0.66
24	—	—	1.85	0.30	0.84

By increasing the amount of enzyme present in the solution the fractional inhibition i has thus been reduced from 0.84 to 0.66. That this reduction is due to a redistribution of the inhibitor, *i.e.*, to the establishment of a new equilibrium, is, moreover, apparent from the slow development of the inhibitory action of the prostigmine after the addition of further enzyme.

The presence of uncombined prostigmine in the inhibited solution has also been proved by ultrafiltration. A portion of the solution with $i = 0.85$ prepared for the preceding experiment was ultrafiltered through a collodion membrane. The ultrafiltrate (2 cc.), which had become somewhat concentrated owing to evaporation, was then mixed with 0.1 cc. of a solution of choline-esterase, a control consisting of 2 cc. water with 0.1 cc. of enzyme being prepared at the same time. Activity determinations after 24 hours gave: control, 3.05; enzyme + ultrafiltrate, 0.75; whence $i = 0.75$. This result clearly demonstrates the presence of uncombined inhibitor in the dispersion medium and hence points to the reversible nature of the combination between enzyme and inhibitor.

The best demonstration of the reversible nature of the process is, however, provided in another manner. It follows from equation (1) that if the concentration of inhibitor be plotted against the inhibition the points should fall on an hyperbola, the line $i = 1$ representing one asymptote. Such a curve has been constructed from the experimental data given in a later section and is reproduced in fig. 1. The points lie satisfactorily about the hyperbola which has been drawn. The position of the second asymptote has also been indicated in the figure.

COMPLETE INACTIVITY OF ENZYME-INHIBITOR COMPOUND

The postulate that one molecule of inhibitor combines with one active centre of enzyme is justified by the minute quantity of the former which is effective. There are, nevertheless, two conceivable mechanisms by which the inhibitory action could be produced. The inhibitor could either, as assumed in this work, occupy the points in the enzyme which are concerned in attaching the substrate and so completely inhibit the activity of the enzyme molecule, or it could combine with the enzyme in such a way that combination with the substrate was rendered more difficult

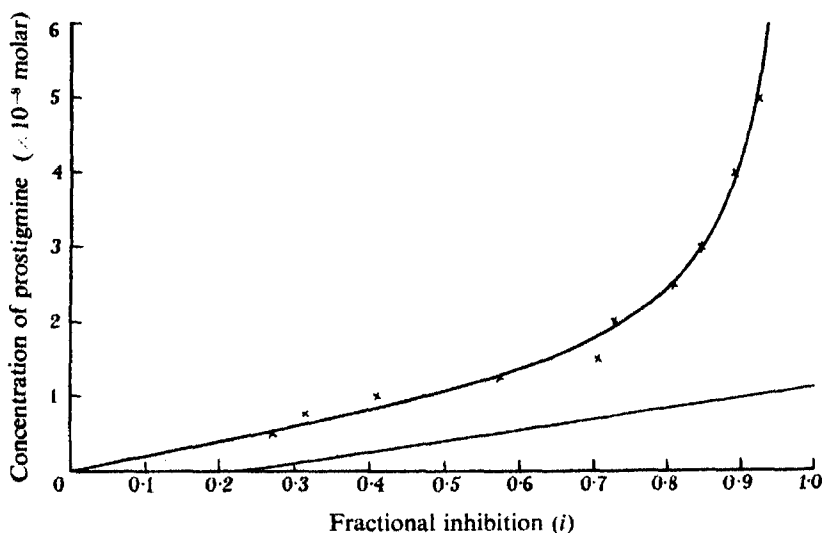


FIG. 1.

but not entirely prevented. In the latter case the enzyme-inhibitor compound would still retain some activity. With the object of deciding between these possibilities, the activity of an enzyme-inhibitor mixture towards different substrates has been examined. If the enzyme-inhibitor compound is completely inactive the magnitude of the inhibition produced by a given concentration of inhibitor should be independent of the substrate. One would not, however, expect this to be so if the alternative mechanism of combination held. In these circumstances the facility with which the various substrates with different spatial arrangements would combine with the enzyme-inhibitor molecule would presumably differ, and hence the inhibitory activity would be dependent on the nature of the substrate. It will be shown below that the inhibitory activity actually remains, within the limits of experimental error, constant under

these conditions, and it can accordingly be concluded that the enzyme-inhibitor compound is completely inactive.

As a preliminary to this work the action of choline-esterase on a number of esters of choline was examined. Stedman, Stedman, and Easson (1932) had already shown that both acetyl and butyryl choline are hydrolysed by this enzyme, and it was of interest, apart from the main object of this investigation, to extend the examination to other esters of choline. The influence of p_H on the rate of hydrolysis has also, to some extent, been studied. The results are collected in Table I, the figures representing the number of cc. of 0.02 N alkali required to neutralize the fatty acid liberated by the enzyme in 20 minutes.

TABLE I—HYDROLYSIS OF VARIOUS ESTERS OF CHOLINE BY 0.5 CC. OF PURIFIED CHOLINE-ESTERASE

Ester	p_H		
	6.8	7.4	8.0
Acetyl choline	2.50	3.40	4.15
Propionyl choline	4.50	5.90	6.85
<i>Iso</i> -butyryl choline	2.95	3.45	3.85
<i>n</i> -butyryl choline	7.10	8.00	8.60
Benzoyl choline	0.75	0.80	0.75

Small corrections have been incorporated in these figures to allow for the non-enzymic hydrolysis of the substrates. These have been determined experimentally under conditions identical with those used in the enzyme experiments except that the alkali employed was only 0.002 N. The results are given in Table II.

TABLE II—NON-ENZYMIC HYDROLYSIS OF VARIOUS ESTERS OF CHOLINE

Ester	p_H		
	6.8	7.4	8.0
Acetyl choline	0.95	2.95	6.05
Propionyl choline	0.70	2.35	5.45
<i>iso</i> -butyryl choline	0.40	1.60	3.90
<i>n</i> -butyryl choline	0.45	1.60	3.80
Benzoyl choline	0.30	1.15	3.35

In employing these results to correct for the non-enzymic hydrolysis of the substrates the figures must, of course, be divided by ten.

As the experiments recorded in Tables I and II are subsidiary to the main purpose of this investigation, some comment may be made on them at this stage. It is immediately evident that the rate of non-enzymic hydrolysis of the various esters depends mainly, if not entirely, upon two factors. These are: molecular weight and hydroxyl-ion concentration. An increase in the size of the acyl group diminishes the rate of hydrolysis, and, since the *iso*-butyryl and *n*-butyryl esters are hydrolysed at the same rate, it is apparently immaterial whether this increase in size is brought about by the lengthening of a normal chain or by the introduction of a side-chain. If the size of the molecule is maintained constant, the rate of hydrolysis is practically proportional to the hydroxyl-ion concentration.

The state of affairs in the enzymic hydrolysis is quite different. Here, an increase in the size of the acyl group increases the rate of hydrolysis unless this increase in size is associated with the introduction of a side-chain, when, in the one case examined, a decrease occurs. Thus the enzymic evidently differs from the non-enzymic hydrolysis in being dependent on the structure of the acyl group. Moreover, the influence of hydroxyl-ion concentration differs with the size and, presumably, nature of the acyl group. With none of the esters examined was an optimum hydroxyl-ion concentration observed within the limits of p_H used. The enzymic hydrolysis of benzoyl choline was uninfluenced by p_H within the range 6.8 to 8.0. With the other esters, however, there are indications that an optimum rate, which lies at a p_H above 8.0, is being approached. It would appear from these results that the influence of p_H on the enzymic hydrolysis is an effect on the enzyme-substrate compound rather than on the enzyme alone.

The inhibitor chosen for the inhibition experiments dealt with in this section was the hydrochloride of the methylurethane of α -*m*-hydroxy-phenylethyldimethylamine, a substance which has been named miotine (Stedman, 1929*b*) on account of its intense myotic activity. This compound appeared to be particularly suitable for the experiments in view, for it possesses a marked inhibitory activity towards liver esterase and also resembles physostigmine closely in its pharmacological properties. In examining its inhibitory action towards choline-esterase, the enzyme preparation was mixed with an equal volume of M/20 phosphate buffer of p_H 7.4. To 10 cc. of the mixture were then added 2.5 cc. of a solution of miotine, containing 1 part of the urethane in 5×10^6 parts of water, and water to bring the volume to 20 cc. A similar solution, but containing no miotine, was prepared as a control. Preliminary experiments had shown that the maximum inhibitory action of the urethane, when used in these small concentrations, was not exhibited until it had been in

contact with the enzyme, in the absence of substrate, for a period of nine hours. The above solutions were therefore kept overnight to allow equilibrium to be attained, after which their enzymic activities towards the different substrates were measured, using 2 cc. of the enzyme-inhibitor mixture or of the control in each experiment. The results are recorded in Table III, from which it can be seen that the inhibition is, within the limits of experimental error, independent of the substrate.

TABLE III—INHIBITION OF CHOLINE-ESTERASE BY MIOTINE

Substrate	Control	Enzyme-inhibitor	% inhibition
Acetyl choline	3.10	1.10	64.5
Propionyl choline	5.35	2.20	62.5
<i>Iso</i> -butyryl choline.....	3.15	1.15	63.5
<i>n</i> -Butyryl choline	7.00	2.70	61.5
Benzoyl choline	0.85	0.30	65.0

APPARENT NON-COMPETITIVE NATURE OF INHIBITION

The inhibition of choline-esterase by the urethanes in question appears to be competitive in the sense that their inhibitory action is developed more slowly in the presence than in the absence of the substrate. When, however, the enzyme and inhibitor have reached equilibrium before the substrate is added, the hydrolysis of the latter, provided a sufficient excess is present, proceeds at a constant rate. This has been found to occur in all of the inhibition experiments carried out in this investigation, and it can therefore be concluded that there is no measurable displacement of the inhibitor during the experimental period.

CHOICE OF INHIBITOR

In view of the necessity of leaving the inhibitors in contact with the enzyme for a period of nine hours, it became necessary to examine their stabilities in aqueous solution. Miotine was first examined in this way. A solution of this substance was prepared in a concentration of 10^{-7} molar and, after four days, its inhibitory activity towards choline-esterase was compared with that of a freshly prepared solution of the same concentration. Using butyryl choline bromide as substrate, the following titration figures were obtained: control, 9.40; with old solution of miotine, 6.90; with fresh solution of miotine, 6.15. The inhibitions were thus 27 and 35% respectively, indicating that the inhibitor had undergone appreciable decomposition during the period of four days.

Similar results were obtained in the more extended series of experiments recorded in Table IV. A 10^{-7} molar solution of *l*-miotine (6 cc.) was mixed with 6 cc. of an enzyme mixture composed of 10 cc. of choline-esterase, 5 cc. of M/20 phosphate buffer, and 5 cc. of water. The enzymic activity of the mixture was then determined in the usual manner at the

TABLE IV—DECOMPOSITION OF MIOTINE

Time after mixing	Titration	% inhibition
Control	6.65	—
h. m.		
1 20	5.20	21.8
3 20	3.80	42.8
6 20	3.15	52.6
23 0	3.40	48.9
43 0	4.35	34.6
95 30	5.95	10.5

times stated, using butyryl choline bromide as substrate. The destruction of the inhibitor is here more rapid than in the previous example. This is no doubt partly due to the presence of the buffer, but it is probable that the effect is to some extent caused by the decomposition of the inhibitor by the enzyme. In any case it is evident that the stability of miotine is not sufficiently great for it to be used in the experiments contemplated.

Attention was therefore directed to prostigmine. This substance is the dimethylcarbamic ester of *m*-hydroxyphenyltrimethylammonium methyl sulphate and was first prepared, following the formulation by one of us of the relationship between chemical constitution and physiological action in this group of drugs, by Aeschlimann and Reinert (1931). Prostigmine was one of a number of esters of disubstituted carbamic esters synthesized by these authors, who also made the important discovery that such urethanes are characterized by much greater stability than the urethanes prepared by Stedman and co-workers, which were esters of carbamic or monosubstituted carbamic acids. The claims of these authors have been fully substantiated in the present work. Thus, no difference could be detected between the inhibitory activities towards choline-esterase of a freshly prepared solution of prostigmine and of one which had been kept for five days. Similar results were obtained in the presence of buffer at p_H 7.4. Thus, 24 cc. of a 2×10^{-8} molar solution of prostigmine were mixed with 6 cc. of M/20 phosphate buffer of p_H 7.4 and the inhibitory action of the mixture tested at intervals. For this purpose, 1 cc. of the enzyme preparation was mixed with 5 cc. of the buffered prostigmine solution and the enzymic activity of the mixture

measured after a period of from 21 to 22 hours. Controls were carried out under identical conditions, using water in place of the solution of prostigmine. The titration figures and inhibitions are given in Table V. There appears to be a small diminution in the activity of the prostigmine

TABLE V—STABILITY OF PROSTIGMINE AT p_H 7.4

Age of solution hr.	Titration		% inhibition
	Control	Experiment	
0	7.05	3.20	54.6
46	6.95	3.15	54.6
95	7.00	3.30	53.0
235	6.20	3.10	50.0

solution after keeping for ten days at p_H 7.4. It is possible, however, that this is connected with the decrease in the activity of the enzyme preparation, as shown by the control, rather than representing an actual decomposition of the prostigmine itself.

These experiments established the suitability of prostigmine, as regards stability in aqueous solution, for the purpose in view. When, however, an examination was made of its behaviour in contact with choline-esterase it was unexpectedly discovered that the urethane was slowly decomposed by the enzyme. This is illustrated by an experiment in which an enzyme mixture, consisting of 2 cc. of a choline-esterase preparation 2 cc. of M/20 phosphate buffer of p_H 7.4 and 8 cc. of a 2×10^{-8} molar solution of prostigmine, was prepared and its enzymic activity, using 2 cc. of the mixture with butyryl choline bromide as substrate, measured at intervals. The results are recorded in Table VI, in which are also given

TABLE VI—ENZYMIC DECOMPOSITION OF PROSTIGMINE

Time after preparation hr.	Control	With prostigmine	% inhibition
20	5.90	1.90	68.0
68	6.50	3.60	44.6
112	5.90	5.20	11.7
141	5.55	5.70	0

the values obtained with a control solution. It is evident from these results that an amount of prostigmine sufficient to produce in 20 hours an inhibition of 68% in the activity of the enzyme with which it was in contact, is completely decomposed by this amount of enzyme in less than six days.

ABSOLUTE RATE OF HYDROLYSIS OF BUTYRYL CHOLINE BY CHOLINE-ESTERASE

A preparation of choline-esterase of weight number 242 (*cf.* Stedman and Stedman, 1935) was employed in this experiment. Mixtures of enzyme and inhibitor were prepared consisting of 2 cc. of the choline-esterase preparation, 2 cc. of M/20 phosphate buffer of p_H 7.4, an appropriate volume of a solution of prostigmine (2×10^{-8} or 10^{-7} molar), and sufficient water to bring the total volume of each mixture to 8 cc. The enzymic activities of the mixtures towards butyryl choline were determined in the usual manner. The essential data are summarized in Table VII.

TABLE VII

Concentration of prostigmine (molar $\times 10^{-8}$)	Time after mixing (hr.)	Titration	Fractional inhibition (i_1 and i_2)	i_2 (corrected for $t_2 - t_1 = 70$)
0	23.25	6.7		
0	95.25	6.85		
0.25	23.25	5.9	0.119	
0.25	94.75	6.2	0.095	0.095
0.75	24.25	4.9	0.269	
0.75	96.0	5.65	0.175	0.173
1.0	26.25	4.35	0.351	
1.0	96.0	5.25	0.234	0.234
1.25	26.75	3.3	0.507	
1.25	96.5	4.5	0.343	0.344
2.0	28.5	2.15	0.679	
2.0	98.0	3.4	0.504	0.505
2.5	28.5	1.5	0.776	
2.5	98.5	2.35	0.657	0.657
3.0	29.5	1.2	0.821	
3.0	98.5	1.8	0.738	0.739
4.0	29.75	0.8	0.881	
4.0	99.0	0.9	0.869	0.869
5.0	30.5	0.55	0.918	
5.0	99.0	0.65	0.905	0.905

In order to determine the numerical values of $k/[E]$ and $K/[E]$ by means of equation (6) it is necessary for the interval $t_2 - t_1$ to be constant throughout the series of experiments. As it was not practicable to arrange this experimentally, a small correction has been applied to the value of i_2 to make this correspond with a value of $t_2 - t_1 = 70$ hours. The magnitude of this correction is, however, exceedingly small and does

not exceed the limits of experimental error. From the values of i_1 and i_2 so obtained, those for $\log i_2/i_1$ and

$$\log \frac{i_1(1-i_2)}{i_2(1-i_1)} + \frac{i_1-i_2}{(1-i_1)(1-i_2)}$$

have been calculated and plotted in fig. 2. An inspection of this figure at once shows that, while the majority of the points fall fairly satisfactorily

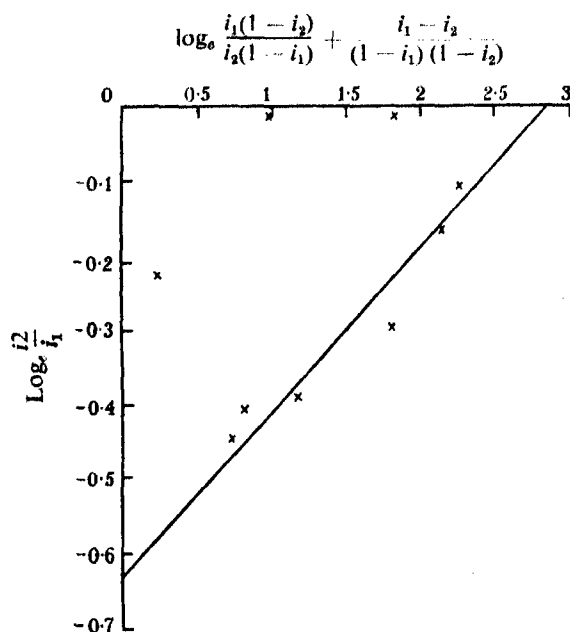


FIG. 2.

about a straight line, there are three which exhibit considerable divergence. We have, however, felt justified in disregarding these points for the following reasons. One of them corresponds with the experiment in which the lowest concentration of inhibitor was employed, and, in view of the small fractional inhibitions produced ($i_1 = 0.119$; $i_2 = 0.095$), must necessarily be subject to considerable experimental error. The remaining two were obtained from the experiments with the highest concentrations of inhibitor. In these cases the inhibitions are so high that they fall in a range where the rate of change of inhibition with change in the concentration of the inhibitor is very small, and are thus also subject to large experimental error. This error is, moreover, considerably magnified by the above treatment. A straight line has therefore been drawn to fit the remaining

points. The intercept on the axis of ordinates is -0.63 , while the slope is 0.22 . Hence, from equation (6),

$$\frac{-k}{[E]}(t_2 - t_1) = \frac{-70k}{[E]} = -0.63;$$

i.e.,

$$\frac{-k}{[E]} = -9.0 \times 10^{-3}.$$

Also $K/[E] = 0.22$. Substituting these values in equation (5), using the limits $(0, 0.95)$, (t, i) , values of t corresponding with various values of i have been calculated and plotted in fig. 3. The curve so obtained, which represents the rate of diminution in inhibition with time, has then been

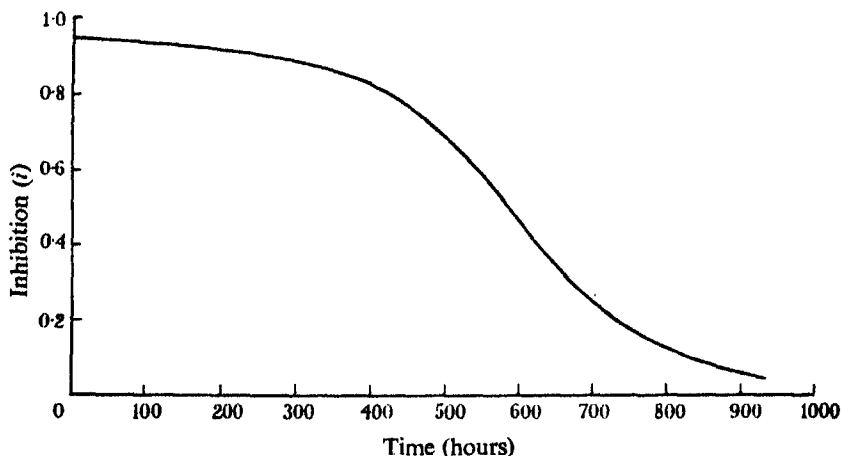


FIG. 3.

employed to correct inhibitions observed at a particular time for the decomposition of the inhibitor which has occurred during its period of contact with the enzyme. For this purpose it is only necessary to find the observed inhibition on the curve, to move back from this point along the abscissa a distance corresponding with the time which has elapsed between mixing the inhibitor with the enzyme and measuring the inhibition, and to read off from the curve the inhibition corresponding with this time. This represents the inhibition which would have been produced had no decomposition of the inhibitor occurred. There is one slight error in this method of correction. The method assumes that combination between enzyme and inhibitor is complete at zero time, *i.e.*, immediately on mixing, whereas this does not happen until some nine hours have elapsed. With the higher concentrations of inhibitor which have been

employed the combination is, however, virtually complete in three hours. The error introduced will therefore be exceedingly small and, when the small relative magnitude of the whole correction is considered, it will be obvious that it is inappreciable.

The observed and corrected values of i_1 have been recorded in Table VIII together with other relevant data. In the same table the calculated values of $1/(1-i)$ and $([I]/i) \times 10^8$ have also been reproduced, while the values of these expressions have been plotted in fig. 4. In conformity with equation (3), the points lie on a straight line. The intercept

TABLE VIII

Time after mixing (hr.)	i_1 (obs.)	i_1 (corr.)	$[I]$ (molar $\times 10^8$)	$\frac{1}{1-i}$	$\frac{[I]}{i} \times 10^8$
23.25	0.119	0.139	0.25	1.16	1.80
19.5	0.239	0.270	0.50	1.37	1.86
24.25	0.269	0.314	0.75	1.46	2.39
26.25	0.351	0.410	1.00	1.70	2.44
26.75	0.508	0.574	1.25	2.35	2.18
27.0	0.649	0.705	1.50	3.39	2.13
28.5	0.679	0.730	2.00	3.70	2.74
28.5	0.776	0.808	2.50	5.21	3.10
29.5	0.821	0.846	3.00	6.49	3.55
29.75	0.881	0.895	4.00	9.52	4.47
30.5	0.918	0.922	5.00	12.82	5.42

on the axis of ordinates is 1.45×10^{-8} while the slope of the line is 0.32×10^{-8} . Hence, from equation (3), $[E] = 1.45 \times 10^{-8}$, while $K = 0.32 \times 10^{-8}$. Since, under the conditions of the experiment and in the absence of inhibitor, 2 cc. of the enzyme mixture hydrolyses in 20 minutes an amount of butyryl choline equivalent to 6.70 cc. of 0.02 N alkali, it follows from this value of $[E]$ that one active centre of choline-esterase hydrolyses in one second

$$\frac{6.70 \times 0.02}{1.45 \times 10^{-8} \times 60 \times 20 \times 2} = 3850 \text{ molecules of butyryl choline.}$$

In order both to confirm the above figure and to ascertain whether its magnitude, as determined by the method described above, is influenced by the impurities necessarily present in the enzyme preparation, two other determinations of the absolute rate of hydrolysis of butyryl choline by choline-esterase have been made. These were carried out with different preparations of the enzyme, one of which was a highly purified sample of

weight number 5050 prepared by adsorption according to the procedure described by Stedman and Stedman (1935), while the second was a less pure specimen, the weight number of which was not recorded. These two preparations yielded values for the absolute rate of hydrolysis of butyryl choline of 3500 and 3160 respectively, figures which confirm that obtained in the experiment recorded in detail above, and hence demonstrate that the presence in the enzyme of impurities, which might conceivably fix some of the prostigmine, does not disturb the method of estimation. It should be mentioned, in connexion with the estimation made with the highly purified preparation, that it was necessary, in this

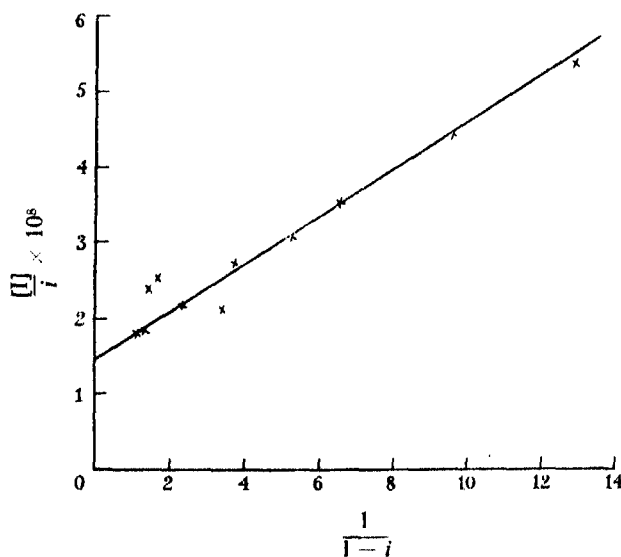


FIG. 4.

case, to restrict the volume of M/20 phosphate buffer employed to 10% of the volume of the enzyme solution. Larger amounts of this buffer produced an inhibition of the enzyme. Purified preparations of choline-esterase are apparently more sensitive than cruder specimens to the action of phosphates.

DISCUSSION

Few estimations have hitherto been made of the absolute rate of activity of an enzyme. The best-founded determination is probably that of Haldane (1931), who calculated from the results of Zeile and Hellström (1930), on the basis of their demonstration that horse-liver catalase is an iron porphyrin compound, that one active centre of this

enzyme destroys 5.42×10^4 molecules of H_2O_2 per second under specified conditions (0°C. and 10^{-2} M substrate concentration), a figure which would probably be increased tenfold at 40°C. , and under optimal conditions of substrate concentration. Kuhn, Hand, and Florkin (1931), on the assumption that peroxidase is also an iron porphyrin compound, have calculated that one active centre of this enzyme uses 10^5 molecules of H_2O_2 per second. Some doubt has, however, recently been thrown by Elliott and Keilin (1934) on their identification of peroxidase with an iron porphyrin compound.

Similar determinations appear to have been made only for two hydrolytic enzymes. Kuhn and Brann (1926) estimate that one molecule of invertase hydrolyses about 200 molecules of sucrose per second at 0°C. , and at optimal p_{H} , their calculation being based on a molecular weight determination of the enzyme by Euler (1922), and on the assumption that the latter's preparation was pure. For the same enzyme, Moelwyn-Hughes (1933) obtains an approximate figure of 7.0×10^3 at 37°C. A different value for the molecular weight, that determined by Euler and Ericson (1922), was, however, assumed, while the invertase preparation of Schneider (1928) was selected as representing the pure enzyme. Much smaller figures have been calculated for pepsin. Using as a basis for his measurements the determination of the molecular weight of the crystalline material, Northrop (1932) finds that the number of equivalents of carboxyl liberated in a second by one molecule of pepsin varies between 0.1 and 16, according to the nature of the protein used as substrate.

The method developed in this communication for the determination of the absolute activity of choline-esterase is of a novel nature, and yields results which relate to active centres rather than to molecules of enzyme, and which are independent of the size of the molecule and of the degree of purity of the enzyme employed. Nevertheless, the value obtained, 3.5×10^3 , is of the same order of magnitude as that calculated by Moelwyn-Hughes for invertase. That it should prove to be much larger than Northrop's figure for pepsin was, perhaps, to be expected, since the substrates involved with the last-named enzyme are of a colloidal nature. It similarly accords with anticipation in being smaller than Haldane's figure for catalase, which is an example of a non-hydrolytic enzyme.

The value of 3.5×10^3 is not, however, a maximum one. The conditions which it was found convenient to choose for our experiments were not quite optimal. At more alkaline reactions the rate of hydrolysis of butyryl choline by choline-esterase is somewhat greater, while further small increases would be caused by the use of higher concentrations of substrate and by raising the temperature from 30° to 37°C. It will be

obvious from Table I that the figures for the other esters examined will be smaller than that for butyryl choline. Thus, for acetyl choline, only $3.4 \times 3500/8.0 = 1490$ molecules would be decomposed in one second at p_H 7.4. Nevertheless, it should be emphasized that the absolute activity of choline-esterase under physiological conditions must be much smaller than this, owing to the minute concentrations of acetyl choline which are involved.

There is one further point to which attention should be directed, namely, our discovery that urethanes of the prostigmine type are slowly decomposed by choline-esterase. An inspection of fig. 3 will show that the rate of this decomposition is exceedingly slow. The experiments which this curve represents were, however, carried out at room temperature. At body temperature the rate of decomposition would doubtless be considerably enhanced and would probably partly, if not completely, explain the short duration of the biological activity of such drugs. This enzymic destruction of the inhibitor has, moreover, enabled us to obtain an independent verification of the various assumptions which have been made in calculating the value of $[E]$ and hence of the absolute activity of the enzyme. Thus, equation (7) yields a value for $K/[E]$ of 0.22. On the other hand, when K and $[E]$ are calculated separately by means of equation (3) values of 0.32×10^{-8} and 1.45×10^{-8} respectively are obtained. The ratio $0.32 \times 10^{-8} / 1.45 \times 10^{-8} = 0.22$, a figure identical with that deduced from the rate of destruction of the inhibitor.

The expenses of this investigation have been met by grants from the Earl of Moray Research Fund of this University.

SUMMARY

The relative rates at which choline-esterase hydrolyses certain esters of choline have been compared with the rates for the non-enzymic hydrolysis. The order for the enzymic hydrolysis is: *n*-butyryl > propionyl > *iso*-butyryl $\approx$ acetyl > benzoyl, while that for the non-enzymic is practically the reverse, namely, acetyl > propionyl > *iso*-butyryl = *n*-butyryl > benzoyl. The rate at which the non-enzymic hydrolysis proceeds is approximately proportional to the hydroxyl-ion concentration and decreases with increase in size of the aliphatic acyl group. In contrast with this the rate of enzymic hydrolysis depends on the structure of the acyl group. With *n*-acids it increases with the size of the acyl group, but the introduction of a side-chain apparently diminishes the rate.

The influence of p_H on choline-esterase to some extent depends on the substrate. For most esters the optimum lies above p_H 8.0. Within the range p_H 6.8–8.0, however, the hydrolysis of benzoylcholine is uninfluenced by p_H .

The inhibitory action of miotine (methylurethane of α -*m*-hydroxyphenylethyldimethylamine) on choline-esterase is constant for the different substrates.

Miotine is unstable in aqueous solution. Prostigmine (dimethylurethane of *m*-hydroxyphenyltrimethylammonium methylsulphate), however, is stable. Nevertheless, both urethanes are slowly decomposed by choline-esterase.

The inhibitory action of prostigmine has been utilized to determine the absolute rate of activity of the enzyme. Under conditions which are nearly optimal, one active centre of choline-esterase hydrolyses 3500 molecules of butyryl choline per second. For acetylcholine the corresponding figure is 1490.

APPENDIX

All of the choline esters employed in the above investigation were prepared in this laboratory. We are therefore adding a few notes, including details of compounds not hitherto characterized, on the methods of preparation.

The esters of choline chloride can be conveniently prepared by Fourneau and Page's method (1914), but a purer product is obtained when equimolecular quantities of trimethylamine and the appropriate ester of β -chloroethylalcohol are heated in benzene solution in sealed tubes at 100° C., for 70–100 hours, rather than at the higher temperature used by Fourneau and Page. With one exception, the requisite esters of β -chloroethyl alcohol were prepared from ethylene chlorohydrin and the corresponding acid chloride. In the case of β -chloroethyl formate, for the preparation of which this method is not applicable, a mixture of formic acid and ethylene chlorohydrin was saturated with dry hydrogen chloride. The product, which was obtained in a yield of 35%, boiled at 130° C., and had $D_4^{20} = 1.2553$. According to Bichowsky (1925), β -chloroethyl formate boils at 94° C., and has $D_4^{20} = 1.17$. That our product was actually the ester in question seems certain from the fact that it yielded, by the above method, 75% of the theoretical amount of formylcholine chloride. (Found: Cl = 21.55; $C_6H_{14}NO_2Cl$ requires Cl = 21.14%.) Our preparation of benzoylcholine chloride had m.p. 204° as compared with 200° given by Fourneau and Page.

With the exception of benzoylcholine chloride, the above chlorides of the esters were not, however, employed in this investigation. In view of the difficulty of purifying them by recrystallization, it was found more convenient to prepare the corresponding bromides. For this purpose, the method used by Renshaw and Bacon (1926) for the production of acetylcholine bromide was used. In place of the sealed tubes used by these authors it was found more convenient to leave the β -bromoethyl ester in contact with a slight excess of a 30% solution of trimethylamine in benzene solution in a cool place for a few days. The bromide of the choline ester, which separated in solid form, was then filtered, washed with benzene, and recrystallized.

β -Bromoethyl formate was obtained as a colourless liquid, b.p. 147–148°, $D_4^{20} = 1.7053$, by refluxing ethylene bromohydrin with a slight excess of formic acid for some hours, the use of a catalyst offering no advantage, and carefully fractionating the product. (Found: Br = 52.47; $C_3H_5O_2Br$ requires Br = 52.22%.)

The remaining β -bromoethyl esters were prepared by saturating with dry hydrogen bromide a mixture of ethylene glycol and a slight excess of the appropriate acid. The product was washed first with water and then with a solution of sodium carbonate and was finally dried with potassium carbonate. The resulting oils were then distilled with little loss. In this way were obtained, in addition to β -bromoethyl acetate:

β -bromoethyl propionate, colourless liquid, b.p. 170–175° (slight decomposition) or 73–74°/16 mm; $D_4^{20} = 1.4589$; yield 59%. (Found: Br = 44.04; $C_6H_{10}O_2Br$ requires Br = 44.14%.)

β -bromoethyl-n-butyrate, colourless liquid, b.p. 82.5°/11 mm.; $D_4^{20} = 1.3730$; yield 67%. (Found: Br = 40.99; $C_8H_{18}O_2$ Br requires Br = 40.98%); and

β -bromoethyl-iso-butyrate, colourless liquid, b.p. 79°/11 mm.; $D_4^{20} = 1.3712$; yield 62%. (Found: Br = 40.94; $C_8H_{18}O_2$ Br requires Br = 40.98%.)

The following data refer to the bromides of the choline esters:

Formylcholine bromide, tablets from absolute alcohol, m.p. 142°. (Found: Br = 37.81; $C_6H_{14}NO_2Br$ requires Br = 37.67%.)

Propionylcholine bromide, prisms from absolute alcohol cooled in a freezing mixture, m.p. 146°. (Found: Br = 33.24; $C_8H_{18}NO_2Br$ requires Br = 33.29%.)

n-Butyrylcholine bromide, crystallizes with considerable loss from methyl ethyl ketone, when it forms hexagonal plates which melt at 100–101° when perfectly dry. (Found: Br = 31.47; $C_{10}H_{20}NO_2Br$ requires Br = 31.45%.) It is very deliquescent.

iso-Butyrylcholine bromide, octagonal plates from alcohol-ether or much acetone, m.p. 127°, is somewhat deliquescent. (Found: Br = 31.35; $C_9H_{20}NO_2Br$ requires Br = 31.45%.)

It was not practicable to study the action of choline-esterase on formylcholine, owing to the rapidity with which the non-enzymic hydrolysis of this ester takes place.

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The Action of Sodium Azide on Cellular Respiration and on Some Catalytic Oxidation Reactions

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INTRODUCTION

Azoimide, HN_3 , was prepared by Curtius in 1890. Loew (1891) studied the effect of this acid and of its sodium salt on a great variety of organisms such as bacteria, moulds, algae, seedlings of plants, ciliates, worms, crustacea, insect larvae, and vertebrates. He found it toxic to these organisms but, while some of them died within 2 or 3 hours in 0·5% solution of this salt, other organisms were killed only after 20–40 hours or even 4–6 days by 0·1% solution of NaN_3 , and ciliates withstood this concentration for even 12 days.

According to Loew, the toxic effect produced by this salt is due to its decomposition accompanied by the liberation of ammonia. This interpretation was not accepted by other workers, and it is now generally accepted that the poisonous effect produced by this substance is due to the

action of the nitrogen anion $\begin{array}{c} \text{N} \\ \diagup \quad \diagdown \\ || \quad \text{N}— \\ \diagdown \quad \diagup \\ \text{N} \end{array}$.

We owe an important study of the physiological action of azoimide to Smith and Wolf (1904). According to these authors, azoimide in intravenous injection affects both nerves and muscles which are paralysed, after a preliminary stage of increased excitability. The vapour of azoimide inhaled causes excitation of the respiratory centres with subsequent paralysis. The acid was found by them to be the most powerful compound containing the trinitrogen group. The introduction of the phenyl radical diminished the effect of the complex. The physiological effect produced by azoimide on muscles or nerves resembles, according to these authors, the action of hydrocyanic acid. Although *in vivo* they did not detect any appreciable effect of azoimide on haemoglobin, *in vitro* they obtained clear indications that it forms a compound with methaemoglobin but not with haemoglobin or oxyhaemoglobin.

Sodium azide was tried, so far, only as a physiological poison, and no attempt has been made to analyse its effect on cellular oxidation reactions.

In 1933 it was shown for the first time (Keilin, 1933, *a*) that sodium azide

inhibits cellular respiration, the oxidation of cytochrome and the indo-phenol reaction in a manner similar to that of KCN. It was also shown (Keilin and Hartree, 1934) that sodium azide strongly inhibits the activity of catalase.

In the present paper it is proposed to give a complete account of the reaction between methaemoglobin and sodium azide, and this will be followed by the study of the action of NaN_3 on cellular respiration and on

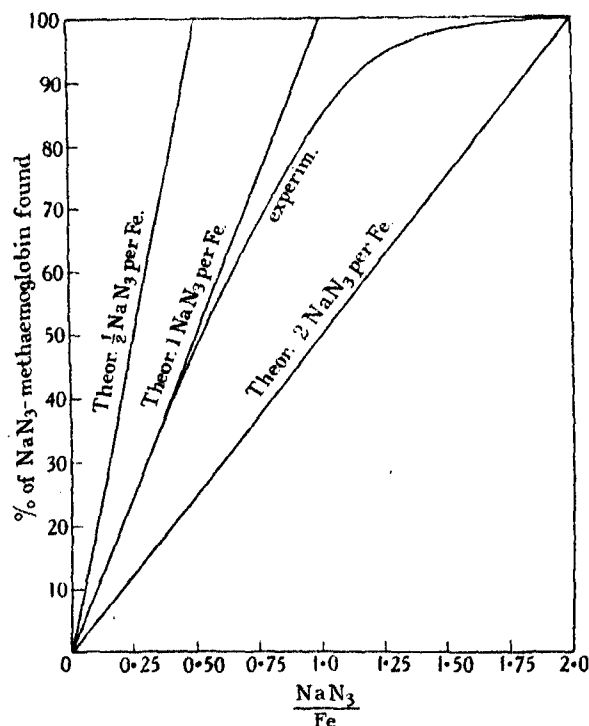


FIG. 1—The formation of NaN_3 -methaemoglobin, showing that the experimental curve lies very near the theoretical curve of 1 molecule of NaN_3 per iron atom of methaemoglobin.

some catalytic oxidation reactions. Throughout this study azoimide was used in the form of its sodium salt (NaN_3), which is very stable and safe to handle.

AZIDE-METHAEMOGLOBIN COMPOUND

The methaemoglobin used in these experiments was prepared from horse blood by the method previously described (Keilin and Hartree, 1935). The solution of this methaemoglobin acidified with KH_2PO_4 to p_{H} 5.6–6.4 is of a brown colour and shows the characteristic absorption

spectrum of acid methaemoglobin. On addition of NaN_3 it rapidly turns red and its absorption spectrum is replaced by two strong but diffuse bands at $575.0 \text{ m}\mu$ and $542.5 \text{ m}\mu$.

For the determination of the minimum amount of NaN_3 which is required to convert acid methaemoglobin to NaN_3 -methaemoglobin, the same method was adopted as was previously described in the study of H_2S -methaemoglobin (Keilin, 1933, *b*). Acid methaemoglobin and sodium azide are mixed in different concentrations in reaction vessels and the relative concentrations of free methaemoglobin and NaN_3 -methaemoglobin formed are determined by comparing the absorption spectrum of the mixture with that given by the two substances; acid methaemoglobin and azide-methaemoglobin in equal compartments of a double wedge trough.

TABLE I

Methaemoglobin as Fe, gm. atom/l	NaN_3 M.	NaN_3/Fe	% NaN_3 methaemoglobin
2.2×10^{-4}	0.38×10^{-4}	0.18	20
2.2×10^{-4}	0.55×10^{-4}	0.25	27
2.2×10^{-4}	0.83×10^{-4}	0.38	33
2.2×10^{-4}	1.1×10^{-4}	0.50	47
2.2×10^{-4}	1.38×10^{-4}	0.63	57
2.2×10^{-4}	1.65×10^{-4}	0.75	68
2.2×10^{-4}	1.94×10^{-4}	0.88	76
2.2×10^{-4}	2.20×10^{-4}	1.00	85
2.2×10^{-4}	2.47×10^{-4}	1.12	89
2.2×10^{-4}	2.75×10^{-4}	1.25	95
2.2×10^{-4}	3.20×10^{-4}	1.45	98

The examination in this case is carried out by means of a Zeiss spectroscope ocular attached to a microscope. The results of these experiments, which are shown in Table I and fig. 1, clearly demonstrate that the minimum amount of NaN_3 required to convert acid methaemoglobin to azide-methaemoglobin corresponds to 1 molecule of NaN_3 per iron atom of methaemoglobin.

The absorption spectrum of azide-methaemoglobin determined in the usual way by means of a Hilger Nutting spectrophotometer is shown in fig. 2. The absorption coefficient is defined here as $\frac{1}{c \times d} \ln \frac{I_0}{I}$, where c is the concentration of methaemoglobin in gram atoms of iron per cubic centimetre, d is the depth of the cell in centimetres, I_0 and I are the intensities of incident and transmitted light which as $\log_{10} I_0/I$ are read off directly on the instrument.

Methaemoglobin combines, therefore, with sodium azide in the same way as it does with KCN, NaF, H_2S , H_2O_2 , and $\text{C}_2\text{H}_5\text{OOH}$.

No compound has been obtained yet between azide and haematin or any other derivative of haemoglobin, apart from methaemoglobin. It may be interesting to note that among the above-mentioned six compounds, which combine with methaemoglobin, KCN alone also forms compounds with other derivatives of haemoglobin, such as haematin, para-haematin, haem, and haemochromogen.

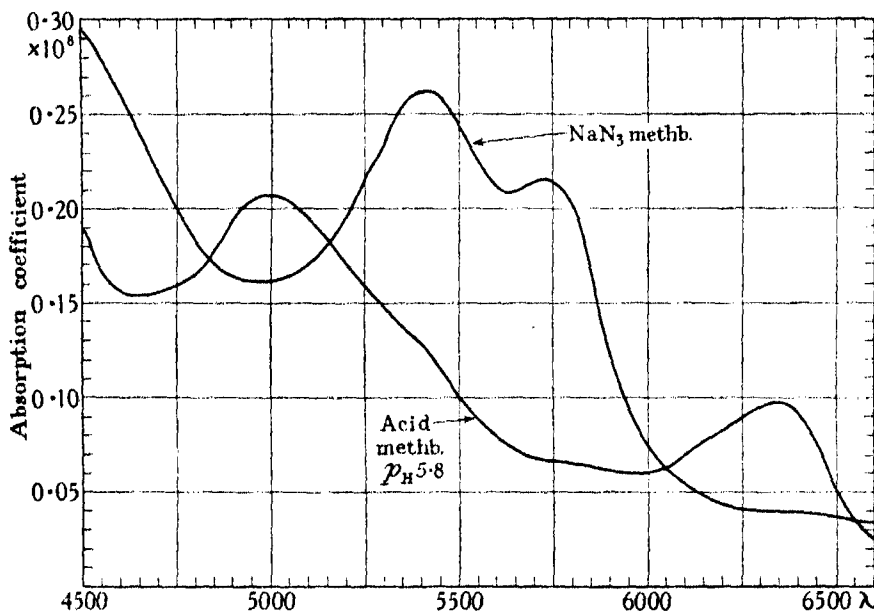


FIG. 2—Absorption spectrum of NaN_3 -methaemoglobin compared with that of acid methaemoglobin. Ordinate, absorption coefficient per gram atom of methaemoglobin iron; abscissa, wave-length in Å.

EFFECT OF AZIDE ON THE OXIDATION OF CYSTEINE BY HAEMATIN

The action of sodium azide on the catalytic oxidation of cysteine by haematin compounds was tested by the oxygen uptake of 8 mg. of metal-free cysteine in presence of 1.6×10^{-4} M. haematin with and without the addition of 0.002 M. NaN_3 .

The oxygen uptakes in 1 hour at room temperature measured in Barcroft differential manometers were as follows:—

	c.mm.
Cysteine alone	14
Cysteine + haematin	205
Cysteine + haematin + NaN_3	162
Cysteine + haematin + $\alpha\alpha^1$ dipyridyl	74

This experiment, repeated several times with various haematin preparations, shows that the inhibition produced by NaN_3 is incomplete; it varies with the haematin preparation from 20 to 50%. Considering that the haematin does not combine with azide, the question arises as to the mechanism of such inhibition. In our experience a haematin solution, when exposed to air, gradually undergoes some irreversible changes which are easily detected, especially when this haematin is transformed into haemochromogen. The absorption bands in this haemochromogen are moved towards the blue end of the spectrum and a new band appears at about 583 $\text{m}\mu$. This modification is accelerated and accompanied by a more or less complete destruction of the haematin when the latter is shaken in air with substances undergoing oxidation.

The iron which still may be combined with the remains of the haematin nucleus may yet have the same catalytic activity towards cysteine as haematin itself. This activity, however, can be inhibited, or completely abolished by the addition of substances which do not combine with the haematin but form catalytically less active compounds with this partly liberated iron. NaN_3 is one of these substances, $\alpha\alpha^1$ -dipyridyl is another.* The latter combines neither with haematin nor with any other iron porphyrin compound but it forms a very stable and catalytically inactive compound with free divalent iron. This explains the strong inhibitory action (64%) which $\alpha\alpha^1$ -dipyridyl produces on the catalytic oxidation of cysteine by haematin. In an experiment of long duration, two hours or more, most of the haematin is completely destroyed and the iron is almost completely recovered as iron- $\alpha\alpha^1$ -dipyridyl compound.

NaN_3 (0.004 M.) also inhibits by 50% the catalytic decomposition of H_2O_2 by haematin. KCN, on the other hand, has no effect, or if anything accelerates this reaction.

THE ACTION OF AZIDE ON PEROXIDASE, CATALASE, AND OXIDASES

Peroxidase—Sodium azide in a concentration of 0.003 M. inhibits by 70% the activity of peroxidase prepared from horse-radish. The same peroxidase preparations are completely inhibited by small concentrations (0.0001 M.) of KCN or H_2S .

Catalase, on the other hand, prepared from horse liver, from blood, or from baker's yeast, is very strongly inhibited by small concentrations of NaN_3 (Keilin and Hartree, 1934). Liver catalase is completely inhibited by 0.002 M. azide, yeast catalase is inhibited by 94% in the

* This inhibitory effect of $\alpha\alpha^1$ -dipyridyl was independently observed by Dr. M. Dixon.

presence of 0.001 M. azide at p_H 6.5. This inhibition, as was shown recently by Blashko (1935), is completely reversible. The mechanism of this inhibition, as well as the action of azide on the haematin compound of the catalase preparation, will be discussed separately in a paper which follows.

Catechol oxidase prepared from cultivated mushrooms and tested with the oxidation of *dl*-dihydroxyphenylalanine was found not to be inhibited by 0.002 M. NaN_3 at p_H 7.3. The same concentration of azide at p_H 5.9 inhibited the activity of the enzyme by 68%.

Indophenol oxidase of muscles (or cytochrome oxidase) is very strongly inhibited by NaN_3 . Thus, 0.001 M. solution of azide, as shown in Table II, inhibits the activity of this enzyme by 80–95%.

NaN_3 inhibits also the oxidation of reduced cytochrome in living yeast cells, but in this case the inhibition depends upon the p_H of the medium. Thus, in yeast cells suspended in phosphate buffer solutions of p_H 8 to p_H 7.5 the effect of azide is hardly perceptible. It begins to act only below p_H 7.3 and in acid phosphate solution its inhibitory effect is as marked as that of KCN.

TABLE II—Effect of NaN_3 on the oxygen uptake in 1 hour by *p*-phenylenediamine in presence of indophenol oxidase of heart muscle, tested in Barcroft differential manometers, containing 3 cc. of solution per flask.

t C°	p_H	Oxidase mg. wet weight	<i>p</i> - phenylene- diamine M	NaN_3 M.	O_2 uptake in c.mm.	% inhibition
18	7.5	200	0.013	—	410	—
18	7.5	200	0.013	0.001	85	80
21	6.5	200	0.013	—	102	—
21	6.5	200	0.013	0.001	21	80
18	7.5	40	0.013	—	68	—
18	7.5	40	0.013	0.001	3	95

ACTION OF NaN_3 ON RESPIRATION OF YEAST CELLS

The study of the effect of NaN_3 on the respiration of yeast cells is carried out in the following way. Fresh samples of baker's yeast are suspended and incubated for at least one hour in phosphate buffer solutions of various p_H and containing 1% of glucose. After incubation, small samples of this suspension, containing from 10 to 20 mg. of wet weight of yeast cells, are put into the right-hand flasks of Barcroft differential manometers. The potash tubes of both flasks receive 0.3 cc.

of 10% KOH and a roll of filter paper, and the left-hand flask receives 3 cc. of the corresponding buffer solution. The oxygen uptake of yeast is then measured in the usual way.

The results of these experiments, which are shown in Table III, clearly

TABLE III—Inhibition by KCN and NaN_3 of oxygen uptake in 1 hour at 18° C. by 10 mg. wet weight of baker's yeast suspended in phosphate buffer solutions containing 1% glucose.

Experiment No.	p_{H}	Inhibitors	O_2 uptake c.mm.	% inhibition
1	7.5	—	151	—
	7.5	0.001 NaN_3	152	0
	7.3	—	164	—
	7.3	0.001 KCN	16	91
	7.3	0.001 NaN_3	144	17
	7.3	0.002 NaN_3	78	55
3	7.3	—	130	—
	7.3	0.001 KCN	12	92
	7.3	0.001 NaN_3	124	5
	7.3	0.002 NaN_3	89	32
4	6.7	—	173	—
	6.7	0.001 NaN_3	43	75
5	6.2	—	134	—
	6.2	0.001 NaN_3	4	97
6	5.6	—	188	—
	5.6	0.001 NaN_3	5	98
7	acid phosph.	—	116	—
		0.001 KCN	3	97
		0.001 NaN_3	12	90
		0.002 NaN_3	0	100
3	acid phosph.	—	156	—
		0.001 KCN	10	94
		0.001 NaN_3	5	98
		0.002 NaN_3	0	100

demonstrate that NaN_3 has a very strong inhibitory action on yeast respiration only if yeast cells are suspended in an acid medium. At p_{H} 5.2–6.2, it inhibits the oxygen uptake of yeast to the same extent as does cyanide; at p_{H} 6.7 the inhibition produced by 0.001 M. NaN_3 is

only 75%; at p_H 7.3, it is only 5–17%; and at p_H 7.5 the respiration is not inhibited at all.

The action of NaN_3 on respiration and different biological catalytic systems is very similar to that of cyanide.

Like KCN and H_2S , it inhibits cellular respiration and the activities of cytochrome oxidase, catechol oxidase, catalase, and peroxidase.

TABLE IV

	KCN	H_2S	NaN_3	CO
Respiration	+	+	+	+
Cytochrome oxidase	+	+	+	+
Catechol oxidase	+	+	+	+
Catalase	+	+	+	+
Peroxidase	+	+	+	—
Uricase	+	—	+	—
Amino acid oxidase	—	+	—	—
Xanthine oxidase	+	—	—	—
Methaemoglobin (Fe^{+++})	+	+	+	—
Haemoglobin (Fe^{++})	—	—	—	+
Haematin (Fe^{+++})	+	?	—	—
Haem (Fe^{++})	+	—	—	+

+ signifies inhibition or reaction with the system.

— signifies no inhibition or no reaction with the system.

Table IV summarizes the effects of the four main inhibitors; KCN, H_2S , NaN_3 , and CO on respiration, the catalytic activities of different enzymes, and the capacity of these inhibitors to react with Fe porphyrin compounds.

SUMMARY AND CONCLUSIONS

The brown solution of acid methaemoglobin on addition of NaN_3 turns red and its absorption spectrum is replaced by two bands at 575 $m\mu$ and 542.5 $m\mu$.

The change in colour and in absorption spectrum is due to the formation of a compound between sodium azide and methaemoglobin.

The formation of azide-methaemoglobin compound requires one molecule of NaN_3 per atom of iron of methaemoglobin.

Like KCN and H_2S , NaN_3 inhibits the activities of indophenol oxidase, of catechol oxidase, of catalase, and of peroxidase. Like KCN, it strongly inhibits the oxygen uptake by yeast cells, but this inhibition depends upon the p_H of the medium. In acid solution at p_H 5.2–6.7 the inhibitory effect produced by NaN_3 is as marked as that produced by KCN; it decreases

with increase in p_H of the solution and at p_H 7.5 the respiration of yeast cells is not inhibited at all.

The inhibitory effect produced by NaN_3 on the catalytic oxidation of cysteine by haematin is probably due to inactivation of some iron derivative of haematin.

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On Some Properties of Catalase Haematin

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INTRODUCTION

It was shown recently by Zeile and Hellström (1930) that a strong preparation of catalase obtained from horse liver has the distinct absorption spectrum of a haematin compound. The concentration of this haematin compound in various fractions of their preparations, estimated as pyridine haemochromogen, was found to be proportional to the enzyme activity of these fractions.

They have also shown that the factors which abolish or inhibit the activity of the enzyme, such as high temperature, alkali, acids, or the addition of small amounts of KCN or H_2S , modify also the absorption spectrum of the haematin.

The haematin nucleus in the catalase preparation was found by them to be similar to that of haemoglobin in forming also a complex compound with a protein. The identity of the haematin nucleus in this compound with protohaematin obtained from haemoglobin was ultimately established by Stern (1936, *a*). According to Zeile and Hellström

(1930) the absorption spectrum of an unmodified catalase preparation shows three bands at I—629 $m\mu$; II—540 $m\mu$; and III—500 $m\mu$. The addition of KCN turns the solution from brown to red and changes the absorption spectrum to two bands at 589 $m\mu$ and 557 $m\mu$. The H_2S derivative of this catalase also gives an absorption spectrum with two bands, but lying, according to these authors, at 640 $m\mu$ and 580 $m\mu$.

To these two derivatives of the catalase haematin Stern (1936, *b*) has recently added a third, which is a compound of this haematin with ethyl hydroperoxide showing two absorption bands at 570 $m\mu$ and 534.5 $m\mu$.

The most remarkable property of the catalase haematin, which has already been noticed by Zeile and Hellström and confirmed by other workers, is that neither $Na_2S_2O_4$ nor potassium ferricyanide has any effect on its absorption spectrum. In other words, it is impossible to ascertain whether the haematin in catalase preparation is a ferric or a ferrous compound.

This property is very unusual for an iron-porphyrin compound which, whether natural or artificial, can always be reduced with $Na_2S_2O_4$ or if not autoxidizable can always be oxidized with potassium ferricyanide, hydrogen peroxide, or potassium permanganate.

In this paper we propose to deal with some of the properties of this remarkable haematin compound and to compare them with those of other and better-known compounds such as derivatives of haemoglobin. This will enable us to determine the valency of the iron in the catalase haematin and also to find the conditions under which the reaction between this haematin and H_2O_2 (its substrate) may be observed spectroscopically.

PREPARATION OF CATALASE AND SOME OF ITS PROPERTIES

Catalase used in this investigation was prepared from horse liver by the method of Zeile and Hellström (1930) slightly modified in order to obtain a strong solution of the enzyme free from alcohol and chloroform (Keilin and Hartree, 1936). The preparation was carried out in the following way.

1400 gm. of fresh horse liver minced into a very fine pulp is mixed with 1400 cc. distilled water, left for one hour, and squeezed through fine linen, giving extract A and the pulp residue. The latter is extracted in a similar way with 1000 cc. of water, giving the extract B. Extract A is treated with half its volume of alcohol (96%) and centrifuged, giving 1260 cc. of dark red liquid which is shaken five minutes with 320 cc. alcohol and 320 cc. chloroform. This manipulation, as has already been shown by Tsuchihashi (1930), completely denatures and precipitates the haemoglobin present in the solution and, on centrifuging, 1150 cc. of greenish-brown fluid is obtained, which is completely free from haemoglobin (solution A_1). This solution is mixed with

7 gm. of freshly prepared gelatinous tricalcium phosphate and centrifuged after 10 minutes. The liquid, which is free from catalase, is rejected while the solid is extracted for 1 hour with 125 cc. of 0.1 M. alkaline phosphate and centrifuged. This extraction is repeated twice, giving in all 325 cc. of a strong brown solution of catalase (solution A₂).

The extract B treated in the same way gives the solution B₁, and, after elution from tricalcium phosphate, another 300 cc. of strong catalase (solution B₂).

These two solutions are purified by ultrafiltration with Bechhold's graded ultra filter membranes. The catalase solution is poured onto a "4½%" membrane, the surface of which is constantly swept by a fine brush driven by a small electric motor. On this membrane the enzyme is rapidly washed with a large amount of water, which removes not only the salts but also all other substances the molecules of which are smaller than those of catalase.

The enzyme can thus be concentrated 10 to 20 times, giving a very dark brown solution, which distinctly shows the absorption band at 629 mμ in a layer 1 mm. deep (solutions A₃ and B₃).

The positions of the absorption bands can be measured with a Hartridge reversion spectroscope adapted as an ocular piece to a microscope. Solutions of catalase are examined in small vertical tubes placed on the microscope stage over the condenser and strongly illuminated by a point-of-light lamp. Measured in this way the 3 main bands occupy the following positions: I—629.5 mμ, II—544 mμ, III—506.5 mμ. The first band, which is the most conspicuous on direct spectroscopic examination, is asymmetric, being stronger on its short wave side. The centre of the band moves therefore, according to the concentration of the pigment, from 627.5 mμ to 630 mμ. This bound haematin compound will be referred to in this paper as native haematin compound.

The total haematin concentration of our preparation is measured by transforming it into pyridine haemochromogen which is compared with a standard solution of haemochromogen prepared from haemin crystals.

The comparison is carried out by means of a Zeiss microspectroscope ocular attached to a microscope. Pyridine haemochromogen obtained from catalase preparation is put into a special Zeiss vessel with a movable plunger while the standard solution of haemochromogen is put into one side of a double wedge trough, (2.3 cm. × 15 cm.), the other side being filled with water. The trough is placed on a platform provided with a scale in front of the comparison prism aperture.

The following is an example of an estimation of total haematin.

1 cc. of strong catalase solution is diluted with water to 10 cc., 4 cc. of this solution is mixed with 0.5 cc. N. NaOH, 0.5 cc. pyridine and a little Na₂S₂O₄. The mixture is put in a Zeiss vessel adjusted to a depth of 1.5 cm.

A standard solution 4.00 mg. of haemin is dissolved in 0.5 cc. of N. NaOH, treated with 10 cc. pyridine, and made up to 100 cc. with water. This solution is reduced with solid $\text{Na}_2\text{S}_2\text{O}_4$ just before putting it into the trough. If the haemochromogen of the standard solution matches that of the catalase preparation when the reading of the position of the trough on the platform is 6.2 cm., the concentration of the haemin in the strong solution of catalase A will be:

$$\frac{6.2 \times 2.3 \times 4 \times 5}{15 \times 1.5 \times 4 \times 100} = 0.314 \text{ mg./cc.}$$

Estimation of Total Iron—A measured volume of catalase solution is heated with 3–4 cc. concentrated H_2SO_4 until acid fumes are evolved. A few drops of potassium chlorate solution are added from time to time until the mixture becomes colourless. The solution is neutralized with ammonia, treated with a little $\alpha\alpha^1$ -dipyridyl and $\text{Na}_2\text{S}_2\text{O}_4$, and the red solution thus obtained is compared colorimetrically with a standard solution of Fe $\alpha\alpha^1$ -dipyridyl.

The catalase activity is defined by Euler and Josephson (1927) as $\text{Cat. f} = k/g$, i.e., the reaction velocity constant (k) divided by the dry weight of the enzyme preparation expressed as gm./cc. The constant k is determined in the usual way: 35 cc. 0.01 N. H_2O_2 is titrated with 0.04 N. potassium permanganate (y cc.). 35 cc. H_2O_2 + 10 cc. 0.02 M. phosphate buffer solution ($p_{\text{H}} = 6.8$) is cooled to 0° and treated with 5 cc. cooled diluted catalase containing 10^{-6} mg. haematin iron. After 5 minutes the reaction is stopped by the addition of 5 cc. 6 N. H_2SO_4 and the residual H_2O_2 determined with the standard permanganate (z cc.). $k = \frac{1}{x} \ln (y/z)$. The oxygen liberated per mg. of dry weight of enzyme per hour at 0° , is calculated from the above permanganate titrations.

The haematin and the total iron content, together with the catalytic activity of several enzyme fractions, have been determined in this way. In Table I are given the results of these determinations for the successive stages B_1 , B_2 , and B_3 of the enzyme preparation previously described (p. 175) and for two other strong preparations A_3 and C_4 which had been washed and concentrated on Bechhold membranes.

This table shows that our enzyme preparations, in addition to the haematin iron, contain other iron compounds which in some very strong preparations represent the main portion of the total iron.

This non-haematin iron appears partly as ordinary metal impurity and partly as a product of decomposition of catalase haematin itself. It is quite possible that the haematin of the catalase preparations when purified

TABLE I.

Enzyme No.	Haematin Fe mg. per cc.	Total Fe mg. per cc.	Dry wt. mg. per cc.	Q_{O_2} = c. mm. O_2		Q_{O_2} = c. mm. O_2		Mols. H_2O_2 decomposed by one mol. haem. per second	Cat. $f = k/g$
				per mg. dry weight per hr. at 0° C.	per mg. haematin per hr. at 0° C.	per mg. haematin per hr. at 0° C.	per mg. haematin per second		
B ₁	0.00029	0.0014	—	—	2.74×10^{10}	2.74×10^{10}	4.4×10^5	—	—
B ₂	0.00168	0.0108	—	—	1.59×10^{10}	1.59×10^{10}	2.5×10^5	—	—
B ₃	0.01410	0.0706	27.4	8.42×10^7	1.31×10^{10}	1.31×10^{10}	2.1×10^5	5.6×10^5	5.6×10^5
A ₃	0.026	1.12	47	16.4×10^7	2.47×10^{10}	2.47×10^{10}	3.9×10^5	8.7×10^5	8.7×10^5
C ₄	0.026	0.51	70	4.9×10^7	1.08×10^{10}	1.08×10^{10}	1.8×10^5	6.2×10^5	6.2×10^5

becomes very fragile and that during its transformation into pyridine haemochromogen it partly decomposes liberating iron.

On addition to such enzyme preparations of small amounts of substances capable of combining with ionized Fe, such as H_2S or $\alpha\alpha^1$ -dipyridyl + $\text{Na}_2\text{S}_2\text{O}_4$ in a slightly acid medium, the solutions turn gradually black or red respectively, until most of the haematin iron is liberated and removed. The activity of the enzyme is, however, not proportional to the total iron but, within certain limits, is parallel to the concentration of haematin iron. It is possible that the enzyme may be damaged or even completely inactivated without affecting the concentration of total haematin estimated as pyridine haemochromogen.

NATIVE HAEMATIN AND TOTAL HAEMATIN CONCENTRATIONS IN CATALASE PREPARATIONS

During the successive stages of catalase preparation several fractions of different grades of purity were collected, and with these fractions we have tried to ascertain whether the whole of the haematin estimated as pyridine haemochromogen derives from the native haematin compound seen in these preparations. In other words, we have tried to determine whether the relative concentrations of pyridine haemochromogen obtained from various catalase fractions are proportional to the concentrations of native haematin compound in the same fractions.

For this purpose we have used a Zeiss microspectroscope ocular attached to a microscope. Taking the concentrations of native haematin and that of pyridine haemochromogen in the strongest sample of catalase as 100, the relative concentrations of these compounds in other fractions can be easily obtained by using the comparison prism of the spectroscope.

Taking into consideration the technical difficulties connected with such determinations, the results obtained (*see* Table II) are surprisingly clear in showing a strict parallelism between the concentrations of native haematin and those of pyridine haemochromogen in various fractions of catalase preparations.

These results corroborate Zeile and Hellström's general conclusion as to the relationship between the enzyme and the haematin compound and definitely establish the fact that the whole of the haematin in our enzyme preparations is present in one bound form showing the characteristic three-banded absorption spectrum. It is also important to note that the enzyme preparations are not only devoid of free unbound haematin but are also free from nitrogenous substances capable of combining with haematin. The addition of free haematin to a catalase preparation does

not modify appreciably its absorption spectrum and does not give rise to haemochromogen in presence of $\text{Na}_2\text{S}_2\text{O}_4$.

THE HAEMATIN COMPOUND IN CATALASE PREPARATIONS FROM
OTHER SOURCES

A haematin compound with a three-banded absorption spectrum similar to that of liver catalase preparation was found by Zeile (1930) in catalase preparations obtained from germinating cucumber seeds. We have prepared catalase from this material, and on concentrating it were able to see the characteristic absorption spectrum of native haematin, thus

TABLE II		
Enzyme fractions	Concentration of native haematin	Concentration of pyridine haemochromogen
1	100	100
2	58	61
3	57	59
4	54	52
5	48	56
6	39	44
7	29	27
8	28	26
9	6	7
10	0	1

confirming Zeile's observation. We have found also that a similar three-banded absorption spectrum can be seen in a catalase solution prepared from red blood corpuscles by the method previously described (Keilin and Hartree, 1936), and concentrated by ultra filtration.

EFFECTS OF VARIOUS SUBSTANCES ON CATALASE HAEMATIN

Having established the fact that our catalase preparations contain only one haematin compound showing the characteristic three-banded absorption spectrum, we shall examine now the effects produced by various substances on this haematin compound. We have selected for this purpose the following substances which either react with, or have an inhibitory effect on, catalase as well as substances which are known to react with methaemoglobin.*

* Catalase haematin, as it will be shown, has several properties in common with methaemoglobin.

(1) $\text{Na}_2\text{S}_2\text{O}_4$, (2) KCN, (3) H_2S , (4) NaF, (5) NO, (6) NaN_3 , (7) NH_2OH , (8) NH_3 , (9) N_2H_4 , (10) H_2O_2 , and (11) $\text{C}_2\text{H}_5\text{OOH}$.

Effect of Sodium Hydrosulphite— $\text{Na}_2\text{S}_2\text{O}_4$, which readily reduces methaemoglobin to haemoglobin, parahaematin to haemochromogen, and haematin to haem, does not affect the absorption spectrum of catalase preparation. If, on the other hand, the mixture of catalase and $\text{Na}_2\text{S}_2\text{O}_4$ is shaken with air, the first absorption band of catalase moves gradually towards the blue end of the spectrum until it reaches the position of $624.5 \text{ m}\mu$. The prolonged shaking with air which completely oxidizes the $\text{Na}_2\text{S}_2\text{O}_4$ does not restore the initial absorption spectrum of catalase.

This in itself shows already that the change in the position of the first absorption band of catalase is not due to the reduction of its haematin nucleus by $\text{Na}_2\text{S}_2\text{O}_4$ but to the appearance of an oxidation product of this reducer. In fact, it can be shown that the same shift in the position of the band can be obtained with sodium bisulphite, SO_2 , or acid buffers, which indicates that the effect produced by $\text{Na}_2\text{S}_2\text{O}_4$ is due to a change in the p_{H} of the solution as a result of oxidation of hydrosulphite.

Effect on KCN and H_2S —These two substances are known as very strong inhibitors of the catalase reaction, and their effects on the absorption spectrum of catalase haematin have been already described by Zeile and Hellström (1930). The addition of a small amount of cyanide to catalase preparation turns its colour from brown to red and its three-banded absorption spectrum is replaced by two bands: α — $595.5 \text{ m}\mu$, β — $556.5 \text{ m}\mu$, the β band being stronger and wider than the α band.

H_2S also forms a compound with catalase haematin, showing an absorption spectrum composed not of two bands as described by these authors, but of three bands having the following positions:

I— $640.5 \text{ m}\mu$ II— $587 \text{ m}\mu$ III— $548 \text{ m}\mu$.

It is important to note that KCN forms well-determined compounds with haem, haematin, and methaemoglobin, while H_2S , as was recently shown (Keilin, 1933), forms a well-defined compound only with methaemoglobin.

Effect of NaF—Sodium fluoride produces only a small inhibitory effect on the activity of catalase. Although it has a comparatively low affinity for methaemoglobin, it combines with it, forming a well-defined fluoride methaemoglobin compound.

The addition of NaF to a slightly acidified catalase solution rapidly changes its absorption spectrum. The first band becomes very narrow,

changes its position to $622\text{ m}\mu$, and a strong wide band appears at $597\cdot5\text{ m}\mu$. This clearly shows that fluoride combines also with catalase haematin.

The small effect it produces on the catalase reaction is probably due to its low affinity for the catalase haematin.

Effect of NO—Nitric oxide combines with methaemoglobin forming a fairly stable compound. The effect of NO on catalase can naturally be tested only in complete absence of oxygen. For this purpose a Thunberg tube containing 3 cc. of strong catalase solution is evacuated, washed several times with pure nitrogen and filled with NO prepared in the usual way from sodium nitrite and acid ferrous sulphate, removing NO_2 with strong sulphuric acid. On shaking the tube, the greenish-brown solution of catalase turns distinctly red and its absorption spectrum is replaced by two strong absorption bands at $577\text{ m}\mu$ and $538\cdot5\text{ m}\mu$, the first band being stronger than the second.

The reversibility of the NO catalase reaction can be demonstrated in the following way. A modified Thunberg tube composed of two branches, one containing catalase solution, and the other a strong solution of sodium sulphite, is evacuated, washed with pure nitrogen, and filled with NO. The tube is shaken until catalase haematin is completely converted into the compound with NO. During 12 hours, the NO is gradually removed from the catalase solution and the absorption spectrum of native catalase-haematin reappears.

Effect of NaN_3 and NH_2OH —Sodium azide, as was previously shown, forms a definite reversible compound with methaemoglobin (Keilin, previous paper). It acts also as a very strong inhibitor of catalase activity (Keilin and Hartree, 1934). On addition of NaN_3 to a strong solution of catalase, it turns slightly more greenish and on spectroscopic examination shows the first band much reinforced and shifted $45\text{--}50\text{ \AA}$. towards the blue end of the spectrum; its final position being $624\cdot5\text{--}623\cdot5\text{ m}\mu$. On washing azide-catalase solution on an ultrafilter, the absorption bands revert to the original intensities and positions. The reversibility of azide poisoning of catalase was recently demonstrated by Blashko (1935).

Hydroxylamine was known so far to react only with haemoglobin, oxidizing it to methaemoglobin. We have seen, however, that the addition of a neutralized solution of hydroxylamine hydrochloride to acid methaemoglobin changes its colour from brown to reddish-brown and modifies the relative intensities of its absorption bands without affecting the general character of the absorption spectrum.

Hydroxylamine is known also as one of the most powerful inhibitors of catalase. When added to a strong solution of catalase it does not modify the general appearance of its absorption spectrum, but it does affect it in some respects. The first band in the red becomes somewhat more diffuse and a shading at $580\text{ m}\mu$, which is hardly perceptible in intact catalase, becomes strong and very distinct.

Although sodium azide and hydroxylamine produce comparatively small changes in the absorption spectrum of catalase, they form, nevertheless, real compounds with the catalase haematin, which, as we shall presently see, acquires new properties.

KCN, when added to fluoride, azide, or hydroxylamine derivatives of catalase haematin, displaces these compounds and gives the KCN derivative. KCN, on the other hand, can be displaced from this compound by the addition of a great excess of any one of these substances, but most readily by azide. This shows that cyanide has a greater affinity for catalase haematin than other substances which combine with it.

Effect of NH_3 and of N_2H_4 —Ammonium hydroxide, added to a strong solution of catalase, distinctly modifies its absorption spectrum. The first band becomes slightly narrower and is shifted to $632\text{--}633\text{ m}\mu$ and two new bands appear at $588.5\text{ m}\mu$ and $550\text{ m}\mu$. That this change in the absorption spectrum of catalase haematin is due to the formation of a compound with ammonia, and not to the change in p_{H} is shown by the fact that a similar change in the absorption spectrum is not obtained on replacing ammonium hydroxide by sodium hydroxide or other alkaline solutions.

The addition of *hydrazine hydrate* to catalase solution produces a similar change in its absorption spectrum, which shows that this substance also reacts with catalase haematin. The effect produced by hydrazine hydrate differs in many respects from that produced by ammonium hydroxide. It will be better understood, however, later on after examination of some other properties of catalase haematin.*

Effect of H_2O_2 and $\text{C}_2\text{H}_5\text{OOH}$ on Methaemoglobin and Catalase—It was shown by Kobert (1900) that the addition of H_2O_2 to acid methaemoglobin changes its colour from brown to red and markedly modifies its absorption spectrum. He ascribed these changes to the formation of an

* The effect of hydrazine hydrate on the absorption spectrum of catalase haematin was noticed independently by Dr. K. Stern who communicated his observation to us. At that time, however, none of us understood the mechanism of the formation of this compound or its real nature.

H_2O_2 -methaemoglobin compound. More recently the existence of this compound was either completely ignored or even denied by other workers.

The formation of H_2O_2 -methaemoglobin was, however, definitely established by Keilin and Hartree (1935) who have also found that ethyl hydroperoxide forms with methaemoglobin a compound very similar to H_2O_2 -methaemoglobin. By spectroscopic and manometric methods they have shown that for the formation of these two compounds one molecule of H_2O_2 or of $\text{C}_2\text{H}_5\text{OOH}$ is required for each atom of iron of methaemoglobin.

These two compounds were found, however, to be very unstable; undergoing rapid decomposition and liberating acid methaemoglobin. It was found (Keilin and Hartree, 1935) that during this decomposition H_2O_2 does not liberate molecular oxygen but seems to be utilized in a peroxidatic reaction. The formation of H_2O_2 -methaemoglobin was ultimately confirmed by Haurowitz (1935) and that of $\text{C}_2\text{H}_5\text{OOH}$ -methaemoglobin by Stern (1935).

Hydrogen peroxide is the only known substrate for the normal activity of catalase, being rapidly decomposed by it into molecular oxygen and water. Ethyl hydroperoxide, on the other hand, is not a substrate, and was found by Stern (1932) to act as a competitive inhibitor of the true catalatic reaction.

It was shown also by Stern (1936, *b*) that ethyl hydroperoxide combines with catalase haematin giving an unstable compound with two absorption bands at $570\text{ m}\mu$ and $534.5\text{ m}\mu$. In this respect catalase shows a still greater resemblance to methaemoglobin.

Ethyl hydroperoxide catalase compound is not affected by $\text{Na}_2\text{S}_2\text{O}_4$ or potassium ferricyanide, but KCN displaces the peroxide and gives a cyan-catalase compound from which cyanide can be displaced, however, by the addition of a great excess of $\text{C}_2\text{H}_5\text{OOH}$.

As to the fate of ethyl hydroperoxide combined with catalase, Stern finds that it is decomposed, yielding acetaldehyde and probably alcohol. That diethylperoxide is decomposed catalytically by an iron salt into aldehyde and alcohol has already been shown by Wieland and Chrometzka (1930). We have demonstrated recently (Keilin and Hartree, 1936) a similar decomposition of ethyl-hydroperoxide by haematin and we have found also that this peroxide when combined with methaemoglobin rapidly decomposes yielding acetaldehyde.

We found no evidence, however, of the formation of acetaldehyde from ethyl hydroperoxide added to a weak solution of catalase, which was completely free from other haematin compounds and from alcohol.

Acetaldehyde was found by us only when ethyl hydroperoxide was

added either to a very strong catalase preparation or to a weak catalase preparation containing a little alcohol.

In this respect it is important to recall that in the presence of a primary oxidation reaction which yields nascent peroxide and of catalase, alcohol always undergoes a secondary or coupled oxidation to aldehyde (Keilin and Hartree, 1936.) The specificity of this reaction makes the explanation of its mechanism somewhat difficult, unless one assumes that the first step is the formation from alcohol and nascent peroxide of an organic peroxide which, on decomposition, yields the aldehyde.

EFFECT OF PEROXIDES ON CATALASE POISONED BY VARIOUS INHIBITORS

We have seen previously that catalase haematin shows a remarkable resemblance to methaemoglobin by forming reversible compounds with KCN, H_2S , NaN_3 , NH_2OH , NaF, NO, and $\text{C}_2\text{H}_5\text{OOH}$, the formation of which can be followed spectroscopically.

Hydrogen peroxide is the only substance which, though it readily combines with methaemoglobin, is rapidly decomposed when added to catalase without affecting the absorption spectrum of the catalase haematin. This result can be explained by assuming that the velocity of decomposition of H_2O_2 by catalase is too great compared with that of the formation of an intermediate compound, so that the life of the H_2O_2 catalase molecule is too short to permit of spectroscopic detection.

The direct spectroscopic observation of this intermediate compound would be possible, however, if the rate of decomposition of H_2O_2 could be slowed down without affecting the formation of the catalase-peroxide compound.

For this purpose we have studied spectroscopically the effect of H_2O_2 on the enzyme poisoned by KCN, H_2S , NO, NaF, NaN_3 , and NH_2OH .

Of these substances NO was found to be the least suitable, because it rapidly oxidized to NO_2 , which destroys the enzyme.

Spectroscopically, KCN-catalase or H_2S -catalase are not affected by the addition of small amounts of H_2O_2 , which shows that KCN and H_2S prevent the reaction between catalase and peroxides and therefore inhibit the formation of an intermediate enzyme-substrate compound. Fluoride-catalase compound is, on the other hand, very unstable and is very rapidly dissociated by the addition of a peroxide. In fact, NaF inhibits the catalatic reaction only when added in very great excess.

Sodium azide and hydroxylamine are the only two inhibitors of catalatic reaction which form compounds with catalase haematin, the reactions of which with peroxides can be observed spectroscopically.

EFFECTS OF PEROXIDES ON AZIDE-CATALASE AND HYDROXYLAMINE-CATALASE

(a)—*Effects of H_2O_2 , BaO_2 , Cerium Peroxide, and C_2H_5OOH* —If we add to a strong solution of azide-catalase a drop of caprylic alcohol and a little H_2O_2 , the latter is rapidly decomposed, but during this process the solution turns distinctly red and the absorption spectrum of azide-haematin is replaced by a spectrum with two bands at $588\text{ m}\mu$ and $547\text{ m}\mu$. When most of the added H_2O_2 is decomposed the colour of the solution turns greenish-brown and the absorption spectrum of azide-catalase reappears. This experiment can be repeated several times with the same sample of azide-catalase. If H_2O_2 is added very slowly, for instance, by dialysing a solution of azide-catalase in celophane against a dilute solution of H_2O_2 , the mixture also turns red and its absorption spectrum is replaced by two strong but less sharply defined bands which lie at $590\text{ m}\mu$ and $554\text{ m}\mu$.

The same compound is obtained when azide-catalase in acid phosphate solution is treated with barium or cerium peroxides or with ethyl hydroperoxide. This clearly shows that azide catalase in presence of different quantities of peroxide forms two distinct compounds.

The spectrum of hydroxylamine-catalase, on the other hand, is not changed in presence of H_2O_2 , which is rapidly decomposed. It reacts, however, with other peroxides; barium-, cerium-, and ethyl hydroperoxide in the same way as azide-catalase, giving a compound with the same absorption spectrum.

We can mention here that the effects produced by peroxides on azide- and hydroxylamine-catalase are more easily obtained in absence of oxygen. This peculiarity will be discussed and explained later in this paper.

(b)—*Effects of Peroxides Formed in Oxidation Reactions*—Sodium hydrosulphite added to azide-catalase produces no change in its colour or its absorption spectrum. On shaking the mixture vigorously with air the solution becomes distinctly red and the absorption spectrum of azide-catalase is replaced by a two-banded spectrum very similar to that produced by BaO_2 . On standing, the colour gradually turns from red to greenish-brown and the absorption spectrum of azide-catalase reappears. On shaking the solution a second time it turns red again, and the two absorption bands reappear. These experiments can be repeated several times while $Na_2S_2O_4$ still remains in the solution. At first sight these experiments seem to show that $Na_2S_2O_4$ reduces azide-catalase in the

same manner that it reduces methaemoglobin or para-haematin. Further analysis of these experiments together with some other tests, however, show that this is not so, and that $\text{Na}_2\text{S}_2\text{O}_4$ does not act as a simple reducer of azide-catalase. The fact that azide-catalase is not affected by $\text{Na}_2\text{S}_2\text{O}_4$ unless the mixture is vigorously shaken with air already militates against this view. This is corroborated by the following experiment:

2 cc. of a strong solution of azide-catalase is put into a modified Thunberg tube, the hollow stopper of which receives 10 mg. of dry $\text{Na}_2\text{S}_2\text{O}_4$. The tube is evacuated and washed out several times with pure nitrogen. On mixing the azide-catalase solution with $\text{Na}_2\text{S}_2\text{O}_4$ and vigorously shaking the mixture, no appreciable change takes place in the colour or absorption spectrum of the solution. If air is now admitted and mixed with the solution, the latter rapidly turns red and the two-banded absorption spectrum appears.

This experiment clearly demonstrates that the changes in colour and absorption spectrum of azide-catalase treated with $\text{Na}_2\text{S}_2\text{O}_4$ take place only in the presence of oxygen. They cannot therefore be ascribed to the reducing action of $\text{Na}_2\text{S}_2\text{O}_4$ but are due to the action of some substance formed during the aerobic oxidation of $\text{Na}_2\text{S}_2\text{O}_4$. That this substance is not bisulphite is clearly shown by the fact that bisulphite does not affect the absorption spectrum of azide-catalase.

The fact that similar changes in the colour and absorption spectrum of azide-catalase can be obtained with various peroxides suggests that the effect produced by shaking azide-catalase with $\text{Na}_2\text{S}_2\text{O}_4$ is also due to a peroxide formed during the oxidation of $\text{Na}_2\text{S}_2\text{O}_4$ by molecular oxygen.

$\text{Na}_2\text{S}_2\text{O}_4$ is not the only substance which displays this interesting property. In fact, other autoxidizable substances such as Stoke's solution (alkaline ferrous sulphate and tartrate) added to azide-catalase and shaken with air turn the catalase solution red and modify its absorption spectrum in the same way as does the addition of $\text{Na}_2\text{S}_2\text{O}_4$ or of various peroxides.

Hydroxylamine-catalase in this respect reacts in exactly the same way as azide-catalase; shaken in air with $\text{Na}_2\text{S}_2\text{O}_4$ or Stoke's solution it turns red and the two-banded absorption spectrum, similar to that obtained with BaO_2 , rapidly appears. These experiments show, moreover, that azide- and hydroxylamine-catalase appear to be the most sensitive reagents for revealing the formation of nascent peroxide in various oxidation reactions.

REDUCTION OF CATALASE HAEMATIN BY PEROXIDES

We have shown so far that peroxides such as H_2O_2 , BaO_2 , cerium peroxide, $\text{C}_2\text{H}_8\text{OOH}$, and nascent peroxide formed during the oxidation

of $\text{Na}_2\text{S}_2\text{O}_4$ or of Stoke's solution, react with azide- or hydroxylamine-catalase and change their colour and their absorption spectra. The problem that now arises is whether the changes produced by peroxides are due to the reduction of haematin iron or reduction followed by compound formation with the H_2O_2 .

That iron can be reduced by H_2O_2 has already been shown by Manchot and Lehmann (1927) and Kuhn and Wassermann (1933). It can be easily demonstrated by mixing a solution of ferric salt and $\alpha\alpha'$ -dipyridyl with H_2O_2 . The divalent iron formed in this reaction rapidly gives the red $\alpha\alpha'$ -dipyridyl compound.

The results of our experiments do not exclude the possibility that peroxides which react or combine with azide-catalase also reduce its haematin iron. This possibility was tested by a series of experiments carried out in modified Thunberg tubes provided with hollow bent stoppers. The catalase solution used in these experiments corresponds approximately to 0.063 mg. catalase haemin per cc. of solution.

Experiment 1—2 cc. of catalase solution mixed with 0.1 mg. NaN_3 and 0.2 cc. of 0.25 M. acid phosphate are placed in the Thunberg tube and 3 mg. of dry BaO_2 are put in the hollow stopper. The tube is evacuated and washed several times with pure nitrogen. At this stage the solution shows the characteristic three-banded absorption spectrum of azide-catalase. On mixing the content of the tube with that of the stopper, the colour of the solution changes to red and the absorption spectrum shows the two distinct bands given by azide-catalase treated with a peroxide. This absorption spectrum remains unchanged for a long time. On letting air enter the tube and shaking the solution, its colour returns to greenish-brown and the absorption spectrum of azide-catalase reappears. On evacuation and washing the tube with N_2 the red colour and the two-banded spectrum reappear.

This experiment clearly shows that the two-banded absorption spectrum belongs to a reduced compound which is reversibly oxidized by molecular oxygen.

Experiment 2—Catalase solution + sodium azide + acid phosphate + BaO_2 are placed in the tube with 0.2 mg. of potassium ferricyanide in the stopper. After evacuation and washing with nitrogen the red compound is rapidly formed. On mixing the contents, there is no change in the colour or the absorption spectrum of the solution.

This shows that the red compound which reacts with molecular oxygen (experiment 1) does not react with potassium ferricyanide. In the same way, it was shown that this compound does not react with $\text{Na}_2\text{S}_2\text{O}_4$.

Experiment 3—Catalase solution + NaN_3 + acid phosphate are put into the tube and BaO_2 into the hollow stopper as in the first experiment. The tube is evacuated and filled with pure CO . Spectroscopic examination of the solution at this stage of

the experiment does not reveal any change in the absorption spectrum of azide-catalase, thus proving that this compound does not combine with CO. On adding the BaO_2 , the mixture turns red but the absorption bands do not occupy their usual positions at $590\text{ m}\mu$ and $554\text{ m}\mu$, as in experiment 1, but are shifted about $100\text{ \AA}$. nearer the blue end of the spectrum and are now at α — $580\text{ m}\mu$ and β — $545\text{ m}\mu$. On opening the tube and shaking its contents with air, the colour and the absorption spectrum revert to those of the azide-catalase compound.

These experiments clearly demonstrate that azide-catalase, when treated with a peroxide in absence of oxygen, forms a red compound which will combine with CO and which is oxidized by molecular oxygen but not by potassium ferricyanide.

Similar results are obtained with hydroxylamine-catalase, which gives the same series of reactions.

The above results apply only to azide- or hydroxylamine-catalase treated either with a very small amount of H_2O_2 or with other peroxides; they do not apply, however, to azide-catalase treated with an excess of H_2O_2 .

Reaction Between Catalase Haematin and Hydrazine Hydrate—We can now submit to a more critical examination, the reaction between catalase haematin and hydrazine hydrate which we have previously described.

The following experiments will help to elucidate this reaction:

Experiment 4—2 cc. of strong catalase solution are put in a Thunberg tube and one drop of 30% hydrazine hydrate is placed in its hollow stopper. The tube is evacuated and washed several times with pure N_2 . After mixing, the absorption bands of catalase haematin become very diffuse and hardly perceptible. On admitting air, two dark bands of almost equal intensity rapidly appear at α — $588.5\text{ m}\mu$ and β — $550.5\text{ m}\mu$ and are followed by the appearance of the band in the red at $629.5\text{ m}\mu$. On evacuating the tube again, the absorption bands disappear almost completely.

Experiment 5—A Thunberg tube is filled in the same way as in experiment 4, but this time the tube is evacuated and filled with pure CO. On mixing the contents, the absorption bands of catalase haematin rapidly disappear, and are not replaced by other bands. On letting in air and shaking the mixture with air and CO, two distinct absorption bands rapidly appear at $579.5\text{ m}\mu$ (α) and $545\text{ m}\mu$ (β), the α band being stronger than β band.*

These experiments show that hydrazine hydrate combines with catalase haematin and in absence of oxygen gives a compound without selective absorption bands. In the presence of oxygen, hydrazine hydrate undergoes oxidation which is accompanied by the formation of a peroxide. The peroxide reacts with catalase haematin, giving a compound with two

* The formation of a CO compound in presence of hydrazine hydrate was also independently noticed by Stern (*see* footnote, p. 182).

absorption bands occupying approximately the same positions as the bands in azide- or hydroxylamine-catalase treated with peroxides. This compound, as was shown in experiment 5, combines also with CO, forming a derivative which has the same absorption spectrum as the CO compound of azide-catalase treated with peroxides.

The action of hydrazine hydrate on catalase haematin in presence of oxygen is therefore comparable to the combined actions of sodium azide and sodium hydrosulphite plus O_2 or sodium azide and a peroxide.

Properties of the Reduced Catalase Haematin—If we accept the view that a haematin which combines with CO and reacts with molecular oxygen is a compound of divalent iron, then azide-, hydroxylamine-, and hydrazine-catalase treated with peroxide should also be considered as compounds of divalent iron. The compound so obtained is either the reduced catalase haematin or a compound between this reduced haematin and H_2O_2 . Azide, hydroxylamine, and hydrazine hydrate form, therefore, with catalase haematin interesting compounds having the following properties: They are not reduced by $Na_2S_2O_4$ but they are reduced by and may combine with peroxides. In the reduced state they combine with CO and although not oxidized by potassium ferricyanide, they are easily oxidized by molecular oxygen.

That H_2O_2 appears to be the only substance which reduces catalase haematin may be due to the fact that it is the only substrate of catalase which reacts with it and probably specifically modifies it in such a way that the iron can change its valency.

It still remains difficult to explain why potassium ferricyanide does not oxidize the reduced form which is so easily oxidized by molecular oxygen. It is, however, conceivable that the first step in the oxidation of the catalase-peroxide compound consists in the reaction between this bound peroxide and molecular oxygen, and, that only when this peroxide is removed by oxidation can the iron of the free catalase haematin undergo oxidation. In order to accept this view we shall have to assume that in the oxidation of this peroxide oxygen only and not potassium ferricyanide can act as a suitable hydrogen acceptor. Such a reaction may be intimately connected with the normal mechanism of catalatic decomposition of hydrogen peroxide.

If we compare various inhibitors of catalase reaction according to the effects they produce on catalase haematin, we can separate them into two categories: (1) Inhibitors like KCN and H_2S which prevent the reaction between catalase and H_2O_2 ; (2) inhibitors like sodium azide, hydroxylamine, or hydrazine hydrate which do not prevent the formation of the

reduced haematin compound, which is possibly an intermediate catalase-peroxide complex, but, on the contrary, stabilize it and in this way inhibit the catalase reaction.

SUMMARY AND CONCLUSIONS

An improved method is described for the preparation from horse liver of a very strong catalase (Cat. $f = 870,000$ and $+Q_{01} = 8 \times 10^7$).

Strong catalase preparations from horse liver, germinated cucumber seeds, or laked blood corpuscles show the characteristic absorption spectrum of a haematin compound described by Zeile and Hellström, having three main absorptions at: I— $629.5 \text{ m}\mu$, II— $544 \text{ m}\mu$, and III— $506.5 \text{ m}\mu$.

The concentrations of this haematin compound in various fractions of enzyme preparation are strictly proportional to the concentrations of total haematin revealed as pyridine haemochromogen.

This shows that the haematin compound with the three-banded absorption spectrum as seen in a fresh catalase preparation represents the total haematin of this preparation.

Observations of previous workers that catalase haematin combines with KCN, H_2S , and $\text{C}_2\text{H}_5\text{OOH}$ are confirmed.

It is shown, further, that catalase haematin combines reversibly with sodium azide, hydroxylamine, sodium fluoride, nitric oxide, ammonia, and hydrazine hydrate, giving rise to spectroscopically well-defined compounds.

Catalase haematin combines, therefore, with all the substances which affect the catalytic activity of the enzyme or which form reversible compounds with methaemoglobin.

On addition of an excess of H_2O_2 to a solution of azide-catalase the colour of the solution turns from greenish-brown to red and the three-banded absorption spectrum is replaced by two bands at $588 \text{ m}\mu$ and $547 \text{ m}\mu$.

When H_2O_2 is added slowly, by diffusing through a celophane membrane, or when other peroxides (BaO_2 , cerium peroxide or ethylhydroperoxide) are added to azide- or hydroxylamine-catalase in acid phosphate solution, the same colour change is observed, but the bands now lie at $590 \text{ m}\mu$ and $554 \text{ m}\mu$.

Similar changes in colour and absorption spectra of azide- or hydroxylamine-catalase are obtained by treating these solutions with $\text{Na}_2\text{S}_2\text{O}_4$ or with Stoke's solution.

It was shown, however, that this change, which takes place only in

presence of oxygen, is not due to $\text{Na}_2\text{S}_2\text{O}_4$ or to Stoke's solution acting directly as reducers, but to H_2O_2 formed during the oxidation of the reducing agents by molecular oxygen.

The action of hydrazine hydrate on catalase haematin is comparable to the combined actions of sodium azide or hydroxylamine and a peroxide, due presumably to the formation of peroxide from hydrazine in presence of oxygen.

Compounds formed in presence of peroxides with azide-, hydroxylamine-, or hydrazine-catalase combine with CO and are oxidized by molecular oxygen.

They are more easily obtained and are stable in absence of oxygen except when oxygen is necessary for the production of the peroxide itself.

These compounds are therefore compounds of divalent iron.

Catalase inhibitors can be separated into two categories: (a) those like KCN and H_2S which prevent the formation of an intermediate reduced catalase-peroxide compound and (b) those like azide, hydroxylamine, and hydrazine, which stabilize the reduced intermediate compound and thus inhibit the catalase reaction.

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Chromosome Behaviour in the Male Ferret and Mole During Anoestrus

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(Communicated by A. S. Parkes, F.R.S.—Received 26 May, 1936)

[PLATES 3, 4]

INTRODUCTION

The breeding season of many Mammals is restricted to a relatively short period of the year. In such species usually both sexes exhibit a more or less well-defined seasonal variation in their reproductive organs. Several investigators have found that these variations in the male consist chiefly of alteration in the size of the testes and epididymis, accompanied by marked histological and cytological changes leading to a complete absence of sperms during the quiescent or anoestrous period.

The cyclic histological changes in the testes and in the accessory organs have been described in detail in the ferret (*Putorius furo*, L.) by Allanson (1932), and in the mole (*Talpa europea*, L.) by Tandler and Grosz (1912) and Allanson (*unpublished*). It was found in both genera that during the breeding season spermatogenesis is complete and very active in the rapidly growing testis, but after that period the reproductive organs return to a quiescent state and sperms are entirely absent.

The present study was undertaken for the purpose of analysing the chromosome complements and behaviour in both genera, with special reference to those changes which lead to the arrest of sperm formation, with a view to determining the cytological basis of the sexual inactivity in the male during the anoestrous period.

MATERIAL AND TECHNIQUE

Testicular material of several ferrets and moles fixed in Bouin's and strong Flemming's solutions was obtained through the generosity of Dr. M. Allanson, Department of Zoology, King's College, London. Some of the animals were killed during the breeding season and others during the non-breeding period, so that the testes under observation exhibited every grade between full activity and quiescent anoestrus.

Sections were cut at 12–15–20 μ . They were stained with Newton's iodine-gentian violet and iron haematoxylin. In both genera the volume of the cells is small ; the chromosomes are very numerous and usually grouped together at mitotic metaphase which makes counting a very difficult task. This difficulty, however, was overcome to a certain extent by strong differentiation in a mixture of clove oil and xylol, in which the slides were left for half an hour or longer. The best results were obtained on slides which were more than a year old, owing to the fact that the stain had faded and the outlines of the individual chromosomes were more distinct. The use of strong Flemming solution had a great disadvantage in that it caused a loosening of the seminiferous tubules and the whole structure of the testes was altered (fig. 12, Plate 3).

The figures were drawn with the aid of a Zeiss camera lucida, using a 20 $\times$ comp. eyepiece and a 1.4 apochr. oil-immersion objective. Most of the drawings were reduced to three-quarters of the original, the magnification being $\times 3500$. Figs. 1 and 10 were drawn at a magnification of 4500.

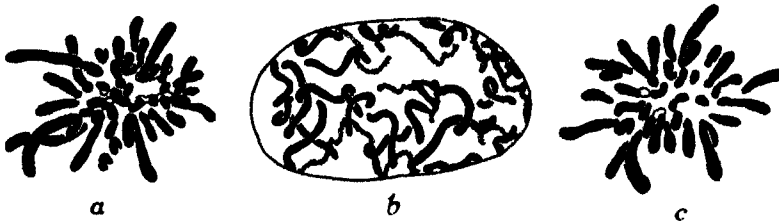


FIG. 1—*a*, spermatogonial metaphase plate of the ferret ; *b*, mitotic prophase ; and *c*, metaphase in the mole.

THE CHROMOSOME NUMBER AND MORPHOLOGY DURING MITOSIS

In moles killed during the non-breeding season it was not possible to find dividing spermatogonial cells suitable for analysis, but a large number of epithelial cells of the epididymis showed mitosis. In the fully active testes of the ferret several spermatogonial divisions were analysed. Both provided sufficient material for the study of the somatic chromosome complements in these genera. The diploid chromosome number in the mole is 38, and in the ferret 34. These numbers were definitely confirmed from a more exact count of the haploid chromosomes in the nuclei during diakinesis.

There is a definite variation in chromosome length ; five pairs in the mole and four pairs in the ferret are long, the others are shorter. The smallest are grouped together in the centre of the equatorial plate (fig. 1*a*, *c*, and fig. 15, Plate 3). The longest chromosomes are not more than 4–5 μ ,

and the smallest about $1.5\text{--}2\mu$. In the ferret the equatorial plate is smaller and the chromosomes are crowded, while in the mole the average volume of the cells is greater. During the late mitotic prophase the chromosomes are evenly distributed in a large oval nucleus, which fills almost the whole cell. (Fig. 1*b* and fig. 14, Plate 3.) The chromosomes show several "relic coils," which are very distinct. The late mitotic prophase was found to be one of the most suitable stages for ascertaining the chromosome number.

Owing to the great number and crowding, it was not possible to determine the shape of all the individual members of the complement. The long chromosomes are V-shaped with unequal arms, and thus have submedian or subterminal centromeres, whilst the smaller are rod-shaped, their centromere being terminal or nearly so.

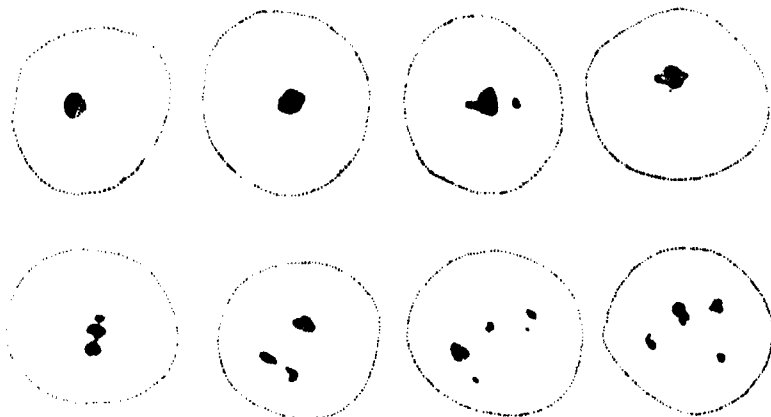


FIG. 2.—Nuclei of primary spermatocytes in the ferret. The nucleolus is breaking up into smaller aggregates.

THE BEHAVIOUR OF CHROMOSOMES DURING MEIOSIS

The spermatogonial cells at the periphery of the seminiferous tubules form one or two uneven layers. Lying amongst the spermatogonia are the Sertoli cells, whose outlines are very indistinct, though they can easily be distinguished by the oval nucleus which contains a small, darkly-stained nucleolus (figs. 20, 21, 22, Plate 4). The fine network of the Sertoli nucleus stains very slightly, but more or less evenly. The nuclei of spermatogonia in the ferret and mole contain several chromatin aggregates of varying size and shape. By analysing a great number of spermatogonia it was found that the chromatin aggregates are derived from the large nucleolus, which gradually breaks up into smaller elements (fig. 2). Dur-

ing the early prophase stages the smaller aggregates rapidly disintegrate, but one or two larger elements persist and disappear only at diakinesis.

During the early prophase stages of meiosis (leptotene-zygotene) the numerous fine chromosome threads form a more or less closely packed bundle. The pachytene is the first stage which can be analyzed in greater detail, because the chromosome number is haploid while the volume of the nucleus has increased. In the ferret, at pachytene the chromosomes form wide closed loops, with the ends lying at an attraction pole, which occupies about one-fifth to one-sixth of the circumference of the nucleus. The

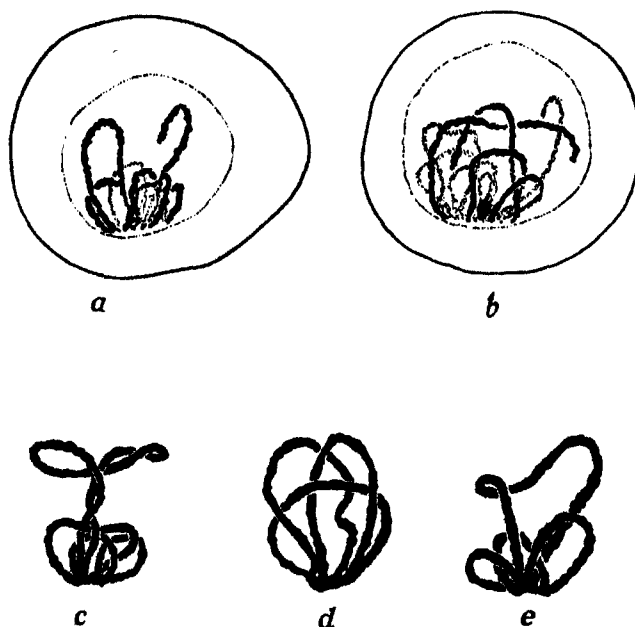


FIG. 3—*a*, early, and *b*, late pachytene stage in the ferret showing polarization ; *c-e*, different configurations of polarized chromosomes.

extension of the loops may be due to repulsion, operating between the paired chromosome threads or to the relational coiling of the homologues. The ends of the threads often appear to be fused together at the attraction pole, which is an artefact caused by fixation. Sometimes the polarized chromosome threads twist and coil round each other (fig. 3*c, d, e*), which may lead to interlocking in the post-diplotene stages. Longer chromosome threads occasionally have only one end at the attraction pole, the other being free and bent towards the centre (fig. 3*a, b*). It should be noted that polarization could be seen in only 12% of the dividing spermatocytes ; this is due to the random orientation of the cells, most of which are

viewed from a position in which polarization, even if present, could not be detected.

During the diplotene stage very few single bivalents could be analyzed. The number of chiasmata in the long bivalents is not less than 4-5 in the ferret, while in the mole it appears to be lower. During the following diakinesis stage the volume of the nucleus greatly increases, the bivalents become more contracted and scattered in the nucleus, and most of the chromatin aggregates disappear. The repulsion which operates between

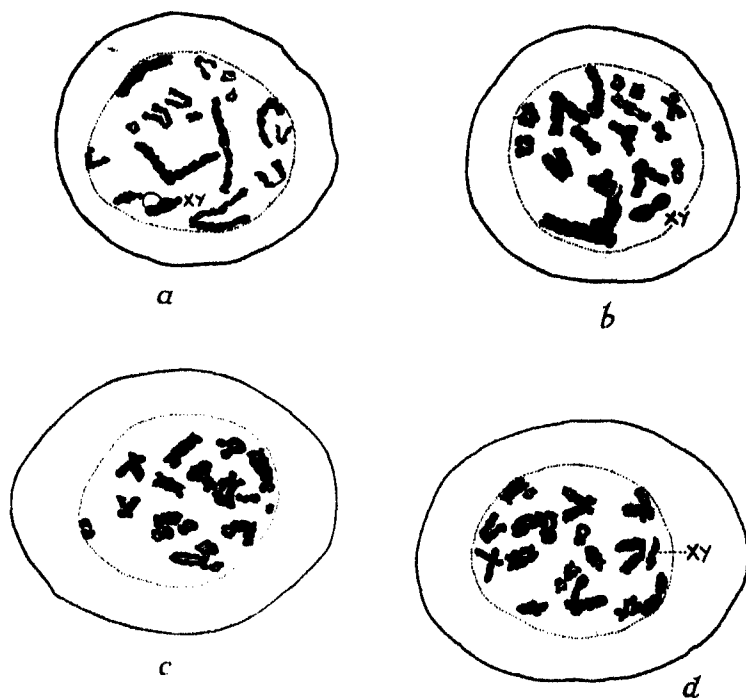


FIG. 4—*a*, early diakinesis in the quiescent testis ; *b*, late diakinesis in the active testis of the ferret ; *c-d*, late diakinesis in the mole.

bivalents at that stage is not very strong, and they are not repelled to the nuclear membrane as in the mouse or rat (Koller and Darlington, 1934). Few bivalents, usually the smaller ones, exhibit various grades of precocious condensation (fig. 4*a-d*). During early diakinesis in the ferret the small nucleolus is still present, and it is frequently found to be associated with a very precocious bivalent, assumed to be the XY or sex-bivalent. The precocity of the XY bivalent is not so pronounced in the mole as in the ferret. During diakinesis the autosomal bivalents show only one or two chiasmata, which are terminal or subterminal, indicating that the terminalization of the chiasmata is high.

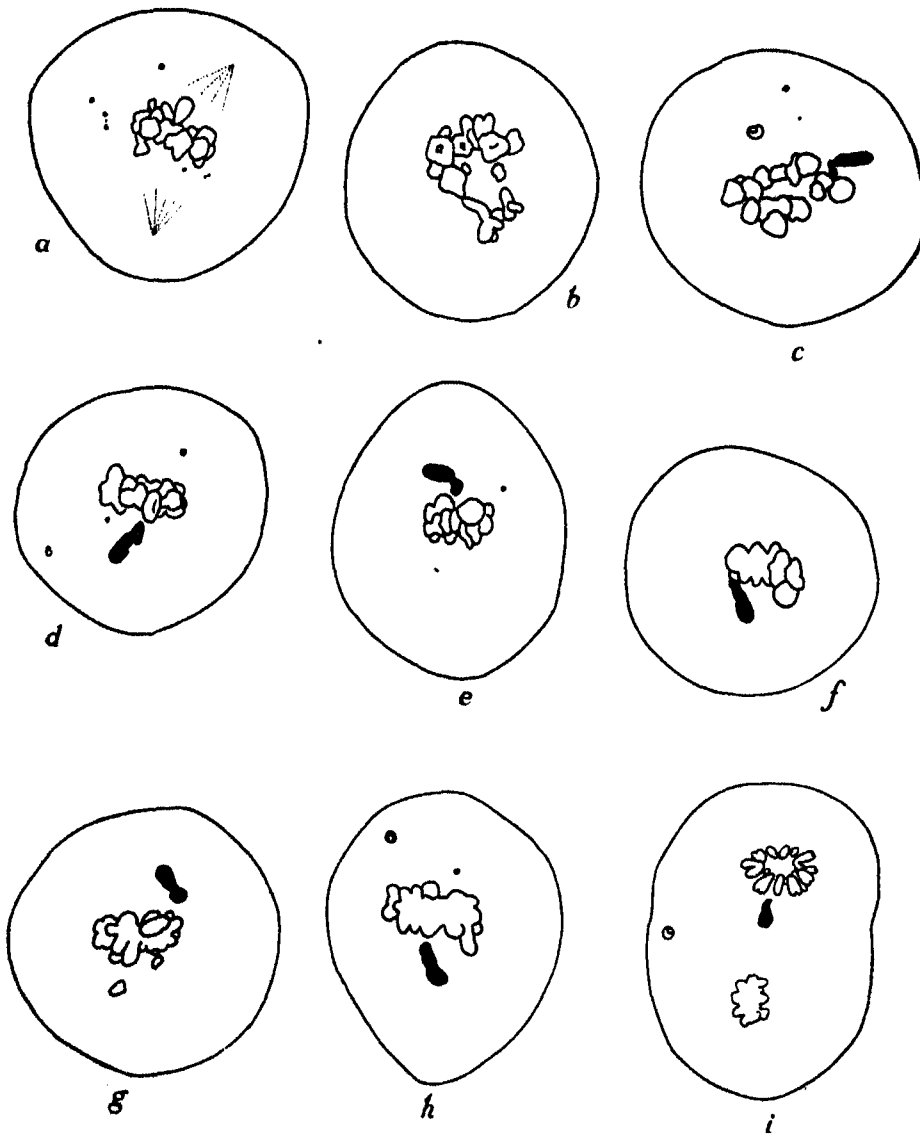


FIG. 5—*a-h*, meiotic metaphase in the ferret—the XY bivalent is black ; *i*, second meiotic metaphase stage in the ferret.

At meiotic metaphase the bivalents of the ferret are grouped at the centre of the cell within a long spindle (fig. 5a). Sometimes the larger bivalents lie off the plate as a result of crowding (figs. 5b, 11a and fig. 18, Plate 3). Several small granules of varying size, which may be seen outside the spindle, represent the remnants of the chromatin aggregates. At present their role in the nuclear cycle is unknown. A chromatoid body is always present; it is larger than the chromatin granules and appears to be composed of two differently stained parts (fig. 5c, h, i). It was found that in the ferret the Y is about 3-4 times smaller than the X chromosome. From the form of the XY bivalent at metaphase it was inferred that in about 32% of the cases the first meiotic division of the sex chromosomes is equational (fig. 5g and fig. 16, Plate 3). The number is very high as compared with the data obtained for the rat (Koller and Darlington, 1934). The high incidence of the equational type of XY bivalent in the ferret suggests that chiasmata are frequently formed in the pairing segment between the centromere and the differential segment (fig. 6).

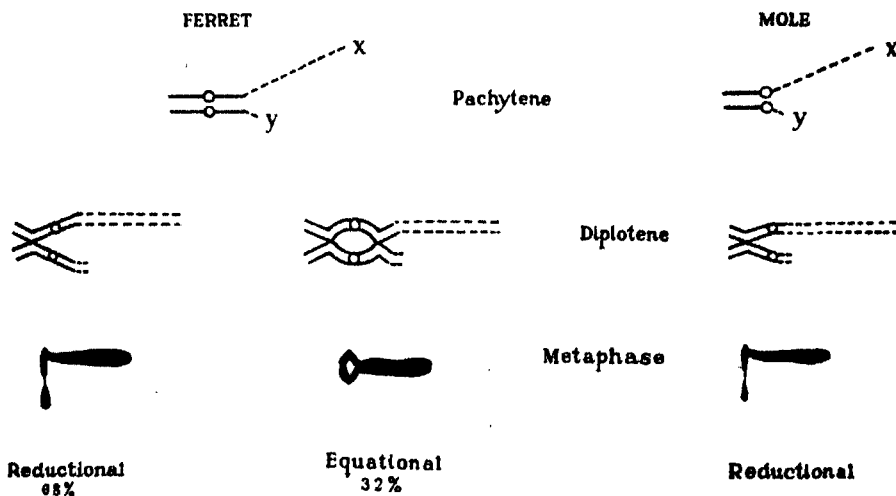


FIG. 6—Diagram illustrating the behaviour of the sex chromosomes during meiosis. Solid line indicates the pairing segment, dotted line the differential segment.

In the mole during first meiotic metaphase the X and Y chromosomes appear to be smaller and thinner than in the ferret; they are not very precocious and the degree of contraction is less (fig. 7a-f). The sex bivalent usually lies outside the equatorial plate, and its shape indicates pre-reduction (fig. 6). Several metaphase plates were analysed, but no equational types were found. This is due to the position of the pairing

segment in the X and Y. It is highly probable that the centromere separates the differential and pairing segments.

Few instances were found in the ferret where the sex chromosomes were left unpaired. They are pushed off the equatorial plate, the small Y chromosome further than the larger X (fig. 22, Plate 3). This behaviour of the sex chromosomes closely resembles that found in *Neuroptera* by Klingstedt (1933).

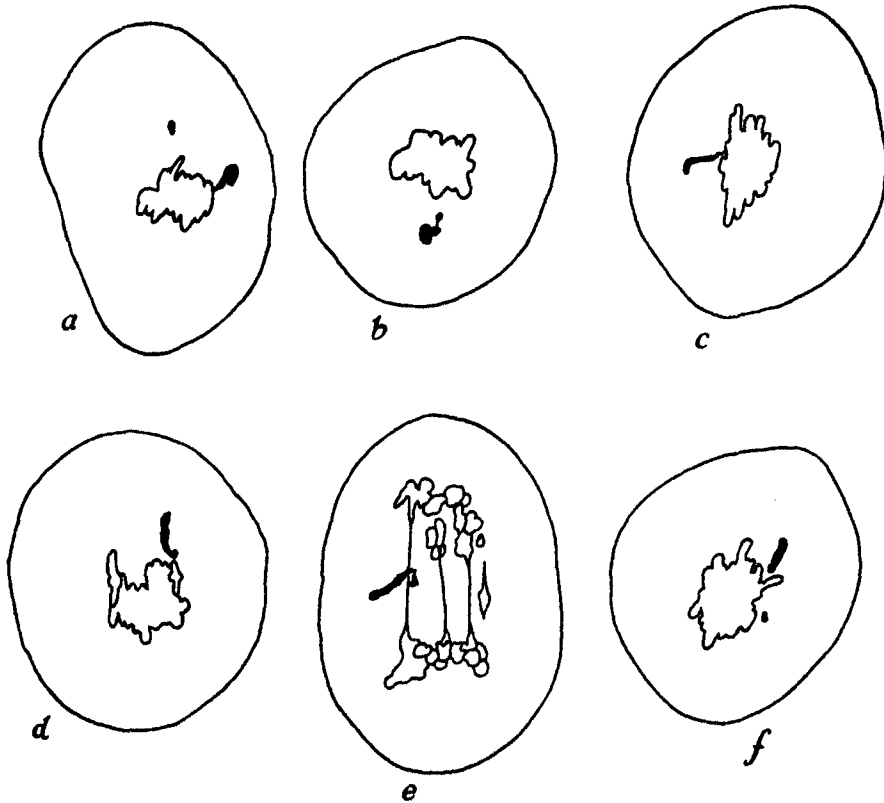


FIG. 7—*a-d*, and *f*, meiotic metaphase ; *e*, first anaphase in the mole. The XY is thin, and its lagging is shown in *e*.

Owing to the crowding of bivalents it was never possible to study the whole chromosome complement. During metaphase only the structure of those bivalents could be analysed which were lying off the equatorial plate, and it was found that the chiasmata are terminal and subterminal, and their number is smaller than at diakinesis. At the first meiotic anaphase the XY bivalent lags behind the autosomes (fig. 7*e*).

The second meiotic metaphase which follows the first anaphase after a short interphase is longer in the mole than in the ferret. The sex chromo-

some very frequently lies outside the equatorial plate as a result of lagging during the previous anaphase (fig. 5*i*).

The secondary spermatocytes in both genera are round. The nucleus is situated at the centre, and has a very distinct and deeply staining network (figs. 22, 23, Plate 4), in which sometimes one or more larger chromatin granules may be present. The early spermatids can easily be distinguished from the secondary spermatocytes by their deeply staining round nuclei (fig. 24, Plate 4). The nuclei of the spermatids gradually become pear-shaped, and the accumulation of post-nuclear granules commences (fig. 25, Plate 4, and fig. 8). Later these granules form a wide, darkly stained band at the base of the nucleus. In the ferret the acrosome is small, and the tail is about 7-9 times longer than the head of the fully developed sperm.

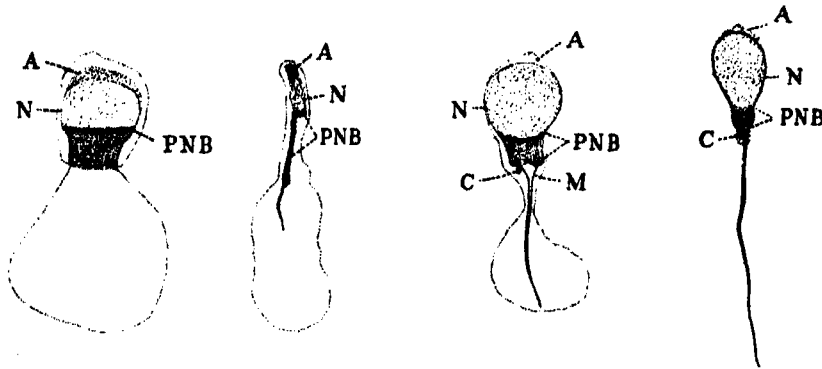


FIG. 8—Spermatoleosis in the ferret. A, acrosome ; N, nucleus ; PNB, post-nuclear band ; C, centrosome ; M, middle part.

CYTOLOGICAL CHANGES IN THE QUIESCENT TESTES

The seminiferous tubules, which during the breeding season are large and contain a well-defined lumen, become much smaller in diameter during the anoestrous period. They become strongly condensed and the lumen completely disappears, the tubules being filled with loose masses of the secondary spermatocytes (figs. 20, 21, Plate 4). The great decrease in the size of the testes of both genera was analysed in detail by Allanson (1932, and unpublished). She found that the weight of the testis varies from 0.178 gm. (November) to 2.062 gm. (April) in the ferret, and from 0.1 gm. (July-December) to 2 gm. (February-April) in the mole. Examination of median cross-sections revealed that in the summer there are fewer tubules in a given area of the testis than during the winter. This is to be expected, since the testis is greatly enlarged, owing to the increased

length and folding of the tubules during the breeding season. A similar observation was made by Allanson (1932).

The cytological changes in the completely quiescent testis were analysed in material from moles killed in November, and the first stages of activity studied in testes from ferrets killed in December and January. It was found that the number of Sertoli cells in the quiescent testes had greatly increased; they were crowded and formed two or three uneven rows. The nuclei of the Sertoli cells are oval, stain lightly with haematoxylin, and contain only one nucleolus. Most of the primary spermatocytes were in the pachytene stage. In the ferret it was found that the frequency of spermatocytes in the pachytene stage had increased to 58% as compared with 36% in the active testis (fig. 9 and Table I). This increase indicates that the pachytene stage is relatively much longer in the quiescent testis than in the fully active one. It is interesting to note that in this stage the paired chromosome threads lie more or less scattered within the nucleus, and complete polarization is rare.

By analysing the structure of the bivalents at diakinesis in the ferret, it became obvious that the number of chiasmata per bivalent is less in the quiescent testis than during the breeding season (fig. 4*a, b*). This difference is even more pronounced in the following metaphase, in which the form of the bivalents has greatly changed owing to the fact that the chromosomes are associated only by terminal chiasmata (fig. 10*a, b*). The smaller number of chiasmata during the non-breeding season may be brought about either by (*a*) a lower initial chiasma frequency in the diplotene stage, or (*b*) a higher terminalization in the post-diplotene stages. If the diplotene and post-diplotene stages of the quiescent testis are of the same duration as the pachytene, which is very long, it may be assumed that the terminalization of chiasmata is more complete during the breeding season. But the very low percentage of primary spermatocytes in diplotene and diakinesis strongly suggests that these stages are relatively short. It is more probable, therefore, that the difference is caused by lower initial chiasma frequency during diplotene. It is generally accepted that chiasma formation is conditioned by genetical crossing-over, hence the lower initial chiasma frequency during the anoestrous period should have an important genetical implication suggesting a decrease in crossing-over between linked genes. Since it is not unlikely that in the future changes in the breeding habits of normally hibernating animals may be induced, the suggestion may possibly prove to be of practical importance.

Spermatids and sperms are entirely absent in the quiescent testes. The secondary spermatocytes, whose further differentiation has been arrested, are very numerous and fill the lumen of the seminiferous tubules. Later

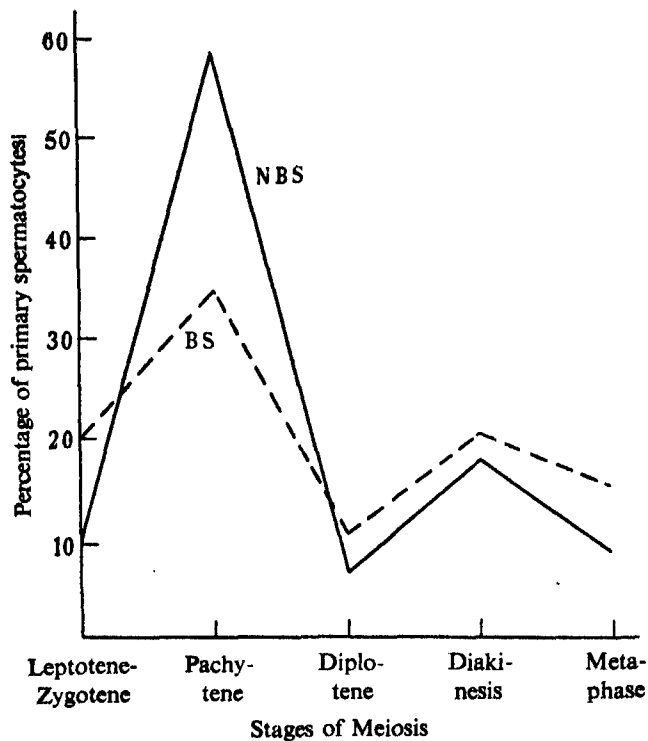


FIG. 9—Graph illustrating the frequency of primary spermatocytes in different stages of meiosis during the breeding (BS) and non-breeding (NBS) season in the ferret.

TABLE I—THE NUMBER OF PRIMARY SPERMATOCYTES IN DIFFERENT STAGES OF MEIOSIS

Stages	Number of spermatocytes	
	Breeding season	Non-breeding season
Leptotene-zygotene	402	203
Pachytene.....	698	1157
Diplotene.....	210	127
Diakinesis	391	353
Metaphase	299	160
Total.....	2000	2000

they break loose from the germinal epithelium. Scattered among them there are occasionally primary spermatocytes, which do not divide and whose nuclei show signs of degeneration.

In the mole several instances were found in which the secondary spermatocytes were divided into two daughter cells, which remained undifferentiated and very closely resembled the parental secondary spermatocytes in every respect. Occasionally binucleate primary and secondary spermatocytes were seen in both genera (fig. 11*b, d*), and a special kind of cell, described by van Beneden (quoted by Courrier, 1927) as "winter spermatogonium," was frequently met with (fig. 11*c, e*). These cells are of three types, viz., mono-, bi-, and multi-nucleate, and their volume is about three times as large as that of normal spermatogonia. The nuclei of these giant

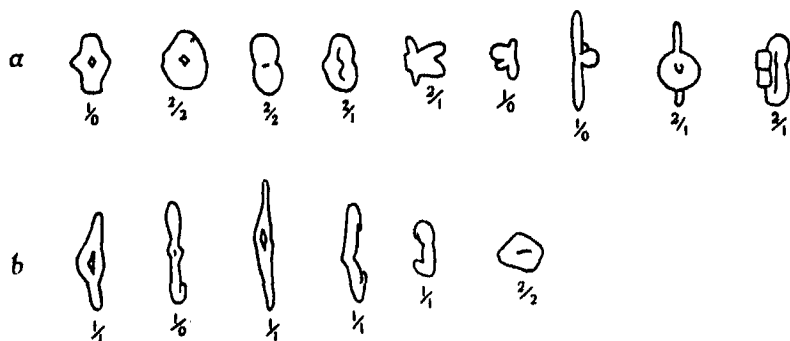


FIG. 10—Metaphase bivalents of the ferret : *a*, in the active, and *b*, in the quiescent testes.

cells contain several chromatin aggregates of varying size. A few instances were found in which the nuclei were packed closely together (fig. 11*c*) ; this would break apart just before the general cellular disintegration. The number of the giant or winter spermatogonia varies, and is correlated with the stage of the anoestrous period, being greater during the early stages than towards the end of this season. In the ferret in the middle of the non-breeding season the giant cells constituted about 3% of the total number of spermatogonia.

The relatively frequent occurrence of binucleate primary and secondary spermatocytes and giant or winter spermatogonia must be considered as a characteristic phenomenon of the spermatogenesis in the quiescent testis. In this respect the testis at this stage is similar to that found in interspecific or intergeneric animal hybrids, such as the Aylesbury $\times$ Muscovy ducks, in which, although chromosome pairing is complete and secondary spermatocytes are produced, no spermatids and sperms are present and the

formation of multinucleate cells is frequent (Koller, 1936, *in press*). The consistent irregularities, as found in the quiescent testis of the mole and ferret, indicate some kind of disturbance in the rate of division and in the time relationship of cytokinesis and karyokinesis, which is probably connected with the physiological changes resulting in sexual inactivity. It

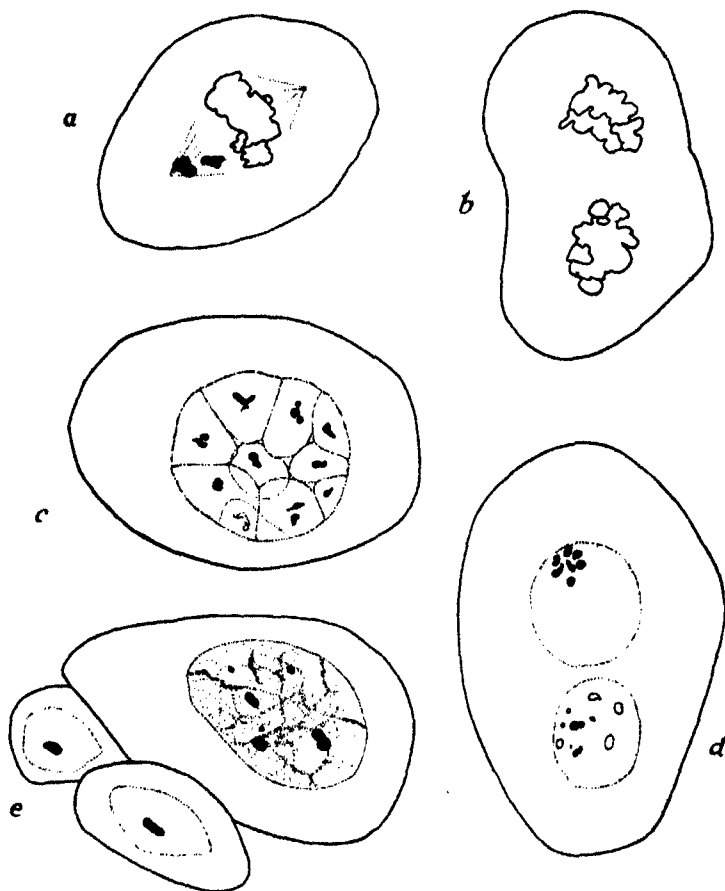


FIG. 11—*a*, meiotic metaphase in the ferret, two bivalents are lying off the equatorial plate ; binucleate spermatocytes, *b*, in the ferret, and *d*, in the mole ; *c* and *e*, giant winter spermatogonia in the ferret.

must be emphasized, however, that although the spermatids and sperms are absent in the quiescent testis, activity does not cease entirely, since primary and secondary spermatocytes are produced in large numbers and the characteristic stages of meiosis are present. My observations thus confirm the statement of Allanson (1932), who suggested "that

possibly there is no period in the cycle of the adult male when activity ceases completely in the seminiferous tubules”.

I wish to express my gratitude to Dr. M. Allanson, who not only provided me with sufficient material and enabled me to undertake this work, but kindly allowed me to see her unpublished data on the reproductive cycle of the male mole, and helped me with valuable suggestions.

The expenses of the original collection of this material were defrayed from grants to Dr. A. S. Parkes from the Foulerton Fund of the Royal Society and from the Medical Research Council.

SUMMARY

The diploid chromosome number in the ferret is 34, and in the mole 38. The chromosomes are of various sizes. In the complement of the male of both genera unequal sex chromosomes are present. The Y chromosome is 3–4 times smaller than the X in the ferret, and about 6 times smaller in the mole.

The sex chromosomes are precocious in their behaviour during meiotic prophase in the ferret. The XY bivalent lies off the metaphase plate in both genera.

The first meiotic division of the X and Y chromosomes is equational in about 32% of the cases in the ferret, but it is invariably reductional in the mole.

In the ferret the chromosome threads are polarized during the pachytene stage.

During the anoestrous period the pachytene stage is prolonged, and binucleate primary and secondary spermatocytes and giant multinucleate cells are frequently met with in both genera.

It is suggested that the initial chiasma frequency at diplotene stage is lower in the quiescent testis of the ferret than in the active one.

During the anoestrous period the secondary spermatocytes do not differentiate further, they degenerate; spermatids and sperms are absent.

The consistent cytological irregularities found in the testes during the non-breeding season indicate a profound disturbance in the rate of division and in the time relationship of cytokinesis and karyokinesis.

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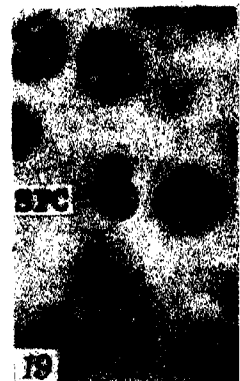
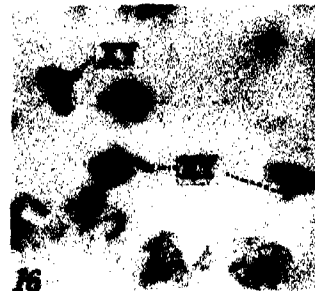
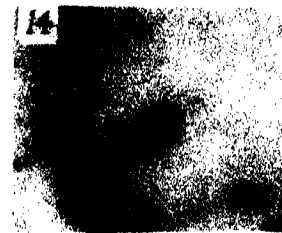
DESCRIPTION OF PLATES

PLATE 3

FIG. 12—Cross-section of the testis of ferret during the breeding and (FIG. 13) during the non-breeding season. FIG. 14—Prophase and (FIG. 15) metaphase of mitosis in the mole. FIG. 16—Meiotic metaphase in the ferret showing the sex bivalent. FIG. 17—Pachytene polarization in the ferret. FIG. 18—Section of the active testis of the ferret (SPC—primary spermatocytes, SPT—spermatids). FIG. 19—Section of the quiescent testis of the mole showing a binucleate secondary spermatocyte with degenerating nuclei.

PLATE 4

FIGS. 20-25—Sections from the testes of the ferret : SN, Sertoli cells ; SPC', primary ; SPC'', secondary spermatocytes ; SPT, spermatids.





The Mechanics of Mitosis in the Pollen-Tube of *Tulipa*

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(Communicated by Sir Daniel Hall, F.R.S.—Received 18 June, 1936)

[PLATE 5]

I—INTRODUCTION

The division of the generative nucleus in the pollen-tube of the higher plants has been studied by several authors, with results differing widely from one another even when concerned with the same genus. For example, Koernicke (1906), Welsford (1914), and O'Mara (1933) have investigated various species of *Lilium*. Koernicke and Welsford describe the formation of a definite though narrow metaphase plate, while O'Mara, in spite of his illustration (fig. 9), is of the opinion that there is no such regular congression owing to the narrowness of the tube. Trankowsky (1930) finds a metaphase plate in *Hemerocallis*, the pollen-tube of which is wide in comparison with the size of the spindle, but not in *Galanthus* or *Convallaria*. Wulff's (1933) observations are weakened by his confusing anaphase with pro-metaphase, and centric with nucleolar constrictions.

A further point at issue is whether the generative cell divides by constriction or by the formation of a cell-plate, the more usual method in the higher plants. Koernicke illustrates a well marked cell-plate, but Wulff and others are of the opinion that division is by constriction.

In view of this conflict of opinion and also of the special spatial conditions obtaining at the pollen-tube mitosis, I have investigated the process in various species of *Tulipa*, both diploid and polyploid, which provide good material for studying the internal as well as the external structure and movements of the chromosomes where varying numbers are present.

The following species were examined :—

<i>T. cretica</i> *		} 2x = 24
<i>T. Greigi</i>		
<i>T. Eichleri</i>		
<i>T. montana</i>		
<i>T. Korolkowi</i>		4x = 48
<i>T. Chusiana</i>		5x = 60

* The authorities for the nomenclature used are given in a complete list of species already published (Upcott and La Cour, 1936).

The ripe pollen-grains were sown on a solution of 14% cane sugar and 2% agar in a Petri dish. When grown in the laboratory at 16°–18° C., germination begins almost at once, but the division of the generative cell is not complete until 24–30 hours after sowing. Small portions of the material were placed on a slide with a needle, and fixed and stained in acetocarmine. The preparations were made permanent by McClintock's method (1929). A few slides were also made by a second method. Material was fixed for two hours in 2 BE (La Cour, 1931) by pouring the fixative on to the surface of the agar. Small pieces were then stripped off with a pair of needles, washed, dehydrated, and stained in gentian violet in a watch glass or small dish. The walls of the pollen-tubes collapse somewhat in the course of dehydration by this method, but the chromosomes are unaffected.

II—EXTERNAL MOVEMENTS

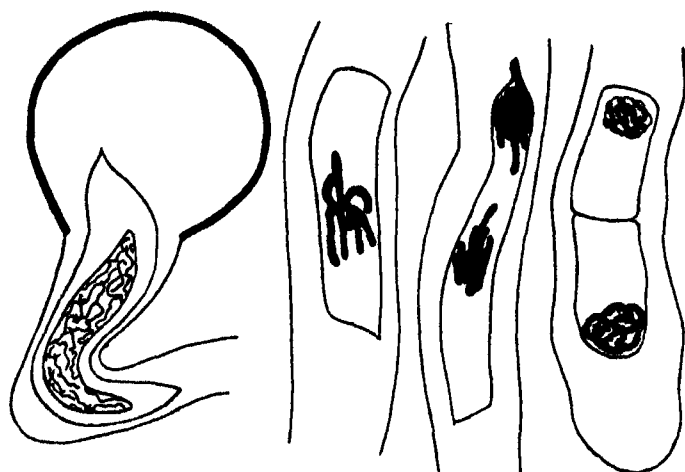
At the time of dehiscence of the anther, the ripe pollen-grain contains two nuclei. One, the tube nucleus, is purely vegetative ; it is large and diffuse, and is concerned only with the growth of the pollen-tube. It never divides, and soon degenerates. The other, the generative nucleus, is smaller and more deeply staining, and divides to form the two male nuclei which bring about double fertilization. This nucleus enters prophase as soon as the pollen-grain germinates. It may be seen at this stage at the mouth of the pollen-tube, two to three hours after sowing. It is surrounded by its cytoplasm, which in acetocarmine preparations is stained more deeply than that of the pollen-tube (fig. 1 and fig. 19, Plate 5). Both the cell and the nucleus are usually rounded at the fore end at this stage, but may be pointed behind. Later, when movement along the tube has presumably slowed down or ceased, the cytoplasm may be pointed at both ends. This, of course, is variable from cell to cell. In gentian violet preparations, the usual number of nucleoli (two or three in *Tulipa*) are visible, but in acetocarmine they are not stained.

Prophase continues for about 20 hours, leading to a stage which may be described as pro-metaphase, in which the nuclear membrane has broken down, but the chromosomes have not yet come on to the equatorial plate. In fixations made between 20 and 24 hours after sowing, this stage is very common and full metaphase rare. I conclude that this is the explanation of the failure of some workers to observe a true metaphase plate.

The chromosomes at pro-metaphase are not yet fully contracted, and still have the irregular outline and relic coiling characteristic of prophase. As metaphase approaches, the spindle may form at one end of the nucleus

so that one group of chromosomes is congressed and orientated on a metaphase plate, while the rest are lying further along the tube, and are thinner and more coiled as at prophase (fig. 12).

The stage between the disappearance of the nuclear membrane and the congression of the chromosomes on the plate is frequently found, and therefore presumably lasts for a considerable time. In pollen-grains and root-tips it is rarely observed, and therefore transitory. The nucleus in the pollen-tube when this stage is observed is often very much elongated. As the spindle forms, therefore, some of the chromosomes will have to travel a considerable distance before they reach the metaphase plate. This does



FIGS. 1-4 *T. montana* ($\times 500$). Drawings of the pollen-tubes photographed in figs. 19-22, Plate 5, showing prophase, metaphase, anaphase, and telophase. Fig. 1 was fixed two hours after sowing, the rest 24 hours.

not always begin to form conveniently within the reach of all the chromosomes, as we have seen. The fact that they have to travel a greater distance would account for the greater length of time required for the complete congression of the chromosomes.

In preparations fixed 24 hours after sowing, metaphases are very frequent, but in others, fixed about two hours earlier or later, they are rare, indicating that the stage is short. Since the nuclei do not develop at exactly the same rate we may assume that the metaphase lasts less than one hour.

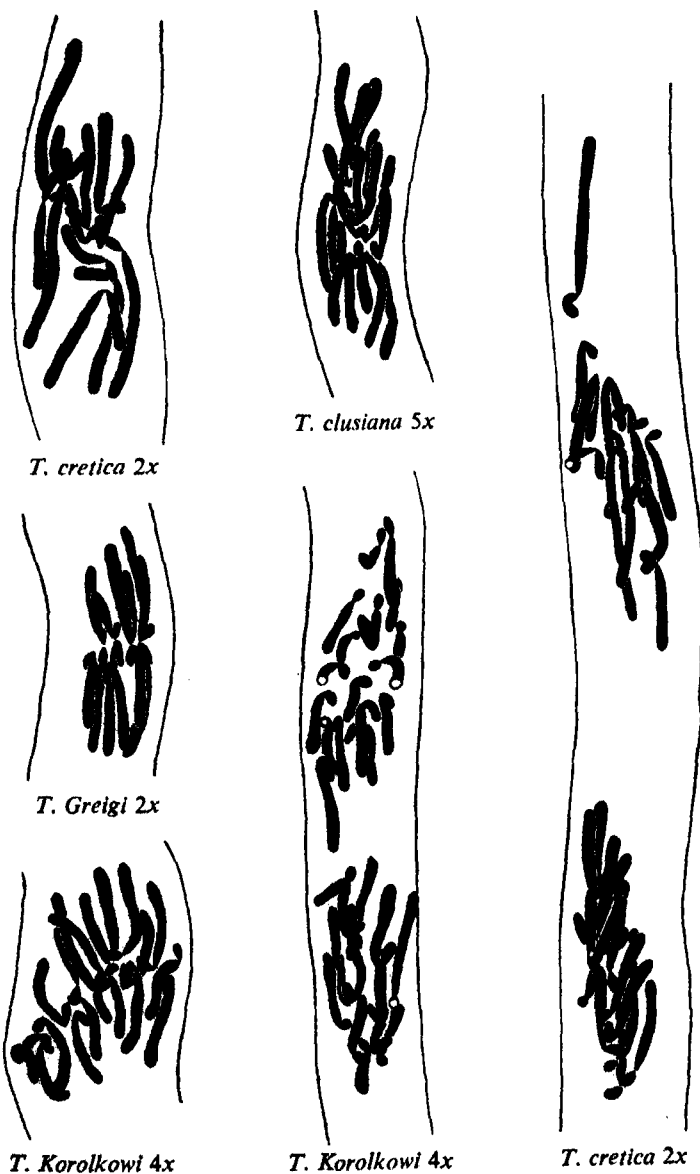
The range of stages in any preparation of a polyploid is greater, owing probably to the fact that many of the nuclei are aneuploid. A metaphase plate is formed in all the species I examined; its shape and position varies according to the size of the pollen-tube and the number of chromosomes

present. It may be formed transversely to the direction of the tube (fig. 6), it may be oblique (figs. 5 and 8), or in extreme cases it may be buckled (fig. 7. *See also* fig. 27). In the diploids, the plate is either transverse or oblique, but in the polyploids it is always oblique and may or may not be buckled. This displacement of the plate has been illustrated without comment by Koernicke, Welsford, and O'Mara in *Lilium*, and observed by Madge (unpublished) in *Hedychium*, which has much smaller chromosomes. It is characteristic of elongated cells in general, for it also occurs in root-tips some distance behind the meristematic region, where the cells are already becoming differentiated.

Owing to the narrowness of the tube the chromosomes are unable to space themselves as they do in unconfined mitoses, but lie with the entire body of the chromosome at right-angles to the plate, so that the centromere is the only part actually on the plate. Yet in spite of this crowding, regular congression on the modified plate is not prevented. It is interesting to notice from acetocarmine preparations that the width of the tube does not directly determine the width of the metaphase plate. There is always a considerable gap on either side of the pollen-tube which is occupied by the cytoplasm of the generative cell (fig. 2 and fig. 20, Plate 5). It would appear, therefore, that the spindle can never push out beyond the bounds of its own cell.

Prophase is not sharply distinguished from metaphase by any particular event, but passes into it by the gradual congression and orientation of the chromosomes on the spindle. Anaphase, on the other hand, is initiated at a definite moment by the division of the centromeres. This leads to their separation for a short distance, pulling the chromatids after them, and undoing thereby any relational coiling that may have survived.

The first stages of anaphase are exactly similar to those found in root-tips or pollen-grains. The later stages are somewhat different, however, owing to the unrestricted opportunities for movement in either direction along the pollen-tube. Early movement is by repulsion of the centromeres (Darlington, 1936, *a*), but later movement, as Belar (1929) has shown, is by stretching of the middle part of the spindle which pushes the newly formed nuclei further apart. In most cells this spindle-expansion is limited by the cell-wall on either side, although in pollen-grains the wall only stops one nucleus, the other being freely pushed into the middle of the pollen-grain (Darlington, 1936, *b*). In the pollen-tube, however, almost limitless growth is possible in either direction. Late anaphases are found, therefore, at very variable distances apart. The generative cell may be compact, with the nuclei separated by a distance of two or three times their own diameter (fig. 3, fig. 21, Plate 5), and the nuclei may even come



FIGS. 5-8—Metaphase in pollen-tube of diploids and polyploids ($\times 1000$). Every chromosome is not represented in the tetraploid (fig. 7) or pentaploid (fig. 8). FIGS. 9 and 10—Anaphase ($\times 1000$), showing isolated chromosomes out of position. The shape of the metaphase plate is still retained.

closer together as they pass to telophase (figs. 4, 14 and 15). At the opposite extreme they may be separated by as much as 30 times their own diameter. In one such case the diameter of each nucleus was 10μ , while the distance between them was 325μ (0.3 mm.). The tube was in this case very narrow.

The orientation and position of the metaphase plate can still be traced from the arrangement of the chromosomes at anaphase. If the plate was transverse the anaphase groups are transverse, while if it was oblique or buckled, the displacement is preserved at anaphase (figs. 9, 10, and 27). This is not surprising, since the greater part of the movement of the chromosomes must be due to the single act of stretching of the middle part of the spindle. It also indicates a remarkable degree of coordination in the timing of separation and in the rate of movement of the chromosomes.

Isolated chromosomes are sometimes found at this stage which may be said to have overshot the mark, and are lying with their distal ends on the opposite side of the pole from the rest of the chromosomes, although their centromeres are not so far out of position (figs. 10 and 13). These chromosomes are found in this position at early anaphase, often before the distal ends of the longer chromosomes have separated. Their position may be due to the sudden separation of the daughter chromosomes which have been coiled, as might occur after release from association in two pieces of elastic under tension. If the subsequent movement of the nuclei is due to the expansion of the central region of the spindle, these chromosomes would be left where they were. Alternatively, they may have been washed out of position by cytoplasmic currents, which are presumably circulating in the generative cell. Their occurrence, however, at the earliest anaphase seems to favour the former explanation.

The individual chromosomes gradually become more indistinct as the nuclei pass to telophase, and the nucleoli reappear at or near the ends of certain chromosomes (fig. 15), corresponding in the two nuclei in size and position. They can be seen only in gentian violet preparations, as at prophase. In these preparations, also, one or more darkly staining bodies appear in the cytoplasm. These vary in number from one to five or six, and also in size. In some cells, where only two are present, they sometimes occur at the poles where the centrosomes should be (fig. 15). Nevertheless, on account of their variability in number and size, it seems improbable that they correspond to the centrosomes, which Guignard and others have reported at this stage.

The limits of the generative cell and of the two male cells into which it divides can be seen by reason of the greater density of the cytoplasm,

which stain more deeply than that surrounding it. When there is a great stretching of the spindle, bringing about a very wide separation of the two telophase nuclei, the cytoplasm does not stain in this way. Its limits cannot be distinguished, perhaps owing to its having been diluted by the great increase in size. In these cases it is impossible to see how the

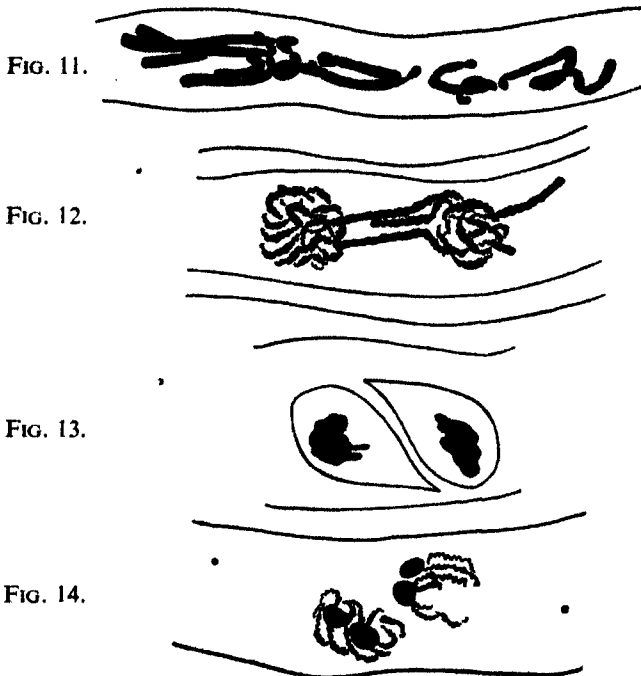


FIG. 11—Pro-metaphase in *T. montana* ($\times 1000$), showing the chromosomes at one end of the nucleus arranged as at metaphase, while those at the other are still in the prophase state. FIG. 12—Telophase in *T. cretica* ($\times 1000$). The two nuclei are held together by two bridges. FIG. 13—Telophase in *T. Greigi* ($\times 500$). An oblique cell-plate has been formed resulting from an oblique metaphase plate. FIG. 14—*T. montana* (fixed in 2 BE and stained in gentian violet. $\times 1000$). Telophase showing the nucleoli and two bodies at the poles, possibly centrosomes. The cytoplasm of the generative cell was not visible, but from the position of the nuclei it is probable that the plate is oblique, as in fig. 13.

cytoplasm separates into two cells, but where the nuclei are close together it is quite clear. An unstained, somewhat refractive cell-plate forms between them (fig. 4 and fig. 22, Plate 5). Its position reflects the original position of the metaphase plate, and, like this, may be transverse or oblique (fig. 13). Presumably the two male cells are always separated in this way, but the cell-plate itself can only be observed where the cytoplasm is dense enough to make it visible by contrast.

III—EXCEPTIONAL BEHAVIOUR

In *T. cretica* four cells out of a large number were observed in which the daughter nuclei were held together at telophase by two chromosome bridges (fig. 13). Such bridges are known to arise at meiosis as the result of crossing-over between two homologous chromosomes, one of which contains a relatively inverted segment. A chiasma in this region gives rise to a dicentric, an acentric, and two monocentric chromatids (Darlington, 1935). In a diploid they usually break at the first or second anaphase of meiosis; in a triploid I have found that they may be preserved to the next division owing to non-disjunction (e.g., in *T. praecox*). In a diploid

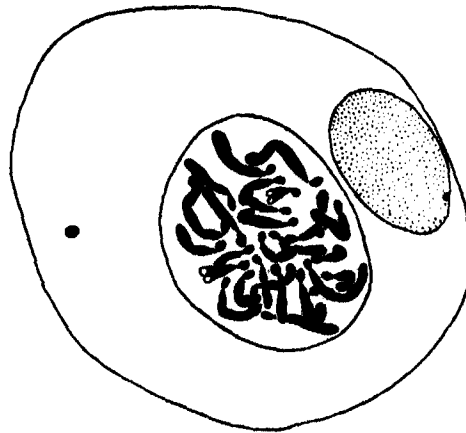


FIG. 15—Division of the generative nucleus while still enclosed within the pollen-grain in *T. Clusiana* ($\times 1000$). There are 28 chromosomes. The tube nucleus is lying against the wall.

such as *T. cretica* it is remarkable that a dicentric chromosome should be preserved unbroken through two divisions. The possibility that some other change took place at the previous pollen-grain division seems to be excluded by the fact that several cells containing such bridges were observed.

Where bridges occur, they are always in pairs and symmetrical, having arisen presumably from a single dicentric chromosome by its division in the previous resting stage. From the mechanical point of view it is interesting to notice that in cells containing bridges the nuclei are always held close together, the possible stretching of the spindle having evidently been prevented.

In most species of *Tulipa* a proportion of living pollen-grains fail to germinate. In these the generative cell may divide to form the two male cells while still enclosed within the pollen-grain. This abnormality occurs

in *T. Clusiana* (fig. 11), a pentaploid in which there is a number of such ungerminated pollen-grains, owing perhaps to the majority of them being aneuploid. It also occurs, though less frequently, in *T. montana*, which is diploid, but which belongs to the same systematic group as *T. Clusiana*.

In the ripe pollen-grain before sowing, the generative cell, whose limits are distinct from the cytoplasm of the tube nucleus, lies close against the pollen-grain wall. The tube nucleus is large and faintly stained, and occupies the middle of the cell. When the generative cell divides, however, this position is reversed. The generative nucleus occupies the middle of the pollen-grain, surrounded by its cytoplasm, and the tube nucleus is pushed to one side (fig. 11). This is probably due to an increase in the size of the generative cell during its period of activity.

IV—INTERNAL MOVEMENTS

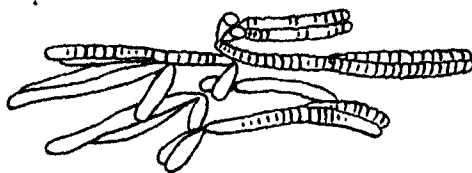
The spiral structure of the chromosomes is most readily demonstrated by acetocarmine preparations of meiosis. At mitosis this is not so easy to see. Pollen-grains can be fixed in acetocarmine, but they have a cell-wall which in many cases modifies the action of the fixative so that the structure is not revealed. Geitler (1935) separated the coils by using acetocarmine followed by pressure, but in many cases the wall is too hard and brittle to allow this method to be used.

As I have already pointed out (Upcott, 1935), the pollen-tube provides a mitosis as readily fixed in acetocarmine as meiosis in the pollen mother-cells, since, like them, it possesses a wall which does not hinder or modify the action of the fixative. At metaphase the fixation varies from cell to cell as it does in pollen mother-cells, but in favourable preparations each chromatid is seen to be composed of spirals, the coils of which have become sufficiently separated from one another to render them visible (fig. 16 and fig. 25, Plate 5). I was not able to determine the direction of coiling at this stage, for the chromatid is only 1.2μ in diameter and the coils are very close together, but the absence of any breaks in the regular succession of coils leads me to suppose that there is no change in direction. The long arm of the chromosome illustrated (fig. 16 and fig. 25, Plate 5) has 33 coils. I was able to make the following measurements :—

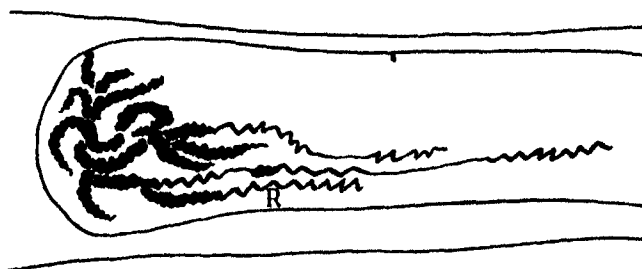
Length of arm of chromosome.....	17.0μ
No. of coils	33
Diameter of chromatid	1.2μ
Calc. diameter of chromosome thread = $\frac{17}{33}\mu$ =	0.52μ
(Wave-length of green light = 0.5μ .)	

Each chromatid is therefore composed of a thread tightly coiled into a cylinder of twice its diameter.

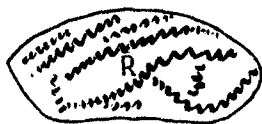
These coils are not usually visible at anaphase, but were accidentally revealed in a damaged nucleus (fig. 17). In this, some of the chromosomes have been pulled out to only a slight extent, so that the direction of the coil is still visible (R). At telophase the spiral structure is particularly clear (figs. 22 and 26, Plate 5). Although it was not possible to make out



T. Creigi



T. montana



T. montana

FIGS. 16-18—Spiral structure ($\times 2000$). Fig. 16, metaphase (cf. fig. 25, Plate 5).

Fig. 17, anaphase. The nucleus has been damaged and the spiral thread uncoiled.

Fig. 18, telophase. The coils at this stage correspond in size and number with those observed at metaphase (cf. fig. 26, Plate 5).

the whole nucleus (fig. 18) owing to the greater indistinctness of the thread as it uncoils, it was possible to determine the direction of the spiral along parts of some of the chromosomes. Moreover, it is evident that the coils seen at telophase are comparable in size and number with those found at metaphase, and must be derived from them merely by a process of loosening.

V—EXTERNAL MECHANICS

The arrangement of the chromosomes on the metaphase plate depends on the shape of the spindle, and the size and number of the chromosomes present. At mitosis the shape of the spindle must be largely determined by the shape and size of the cell within which it is formed. Mitoses in the root-tip, pollen-grain, and pollen-tube should then present an entirely different appearance at metaphase.

This we find to be so. In the pollen-grain (fig. 27, x P.G.) we have a large cell in relation to which the volume of the chromosomes is small.

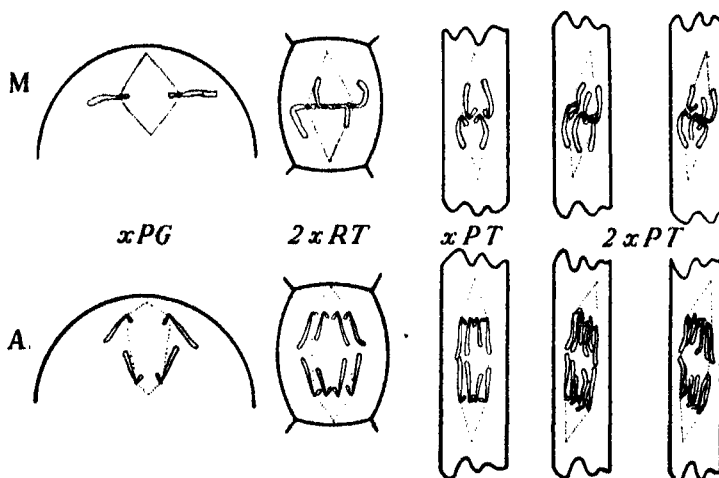


FIG. 27.—Diagram illustrating the effect of the size of the cell and the number of chromosomes present on the shape of the spindle at metaphase and anaphase. PG = pollen-grain, RT = root-tip, PT = pollen-tube, x = haploid, $2x$ = diploid.

One of the poles lies very close to the wall, as we see from asymmetrical anaphases (Darlington, 1936, *b*). Side views of metaphase show that the plate itself is not far from the wall, and if, as we must assume, the two poles are equidistant from the plate, they must be relatively close together. The repulsion from them acting upon the chromosomes must therefore be relatively strong. As the cell is large there is plenty of room for the spindle to expand laterally, so that we get the "cart wheel" appearance characteristic of pollen-grains, in which all or most of the chromosomes are lying flat upon the metaphase plate, usually at the edge of the spindle (fig. 23, Plate 5). The arrangement due to the centrosome repulsion is modified by the fact that the chromosome bodies also repel one another, so that those lying flat round the edge of the spindle are arranged approxi-

mately equidistant from one another. Owing, presumably, to lack of space round the edge, a few chromosomes lie in the centre of the spindle. The body-repulsions prevent them from lying flat, and cause their ends to turn either up or down off the plate. This "hollow spindle" is characteristic of metaphases in which there is a strong polar repulsion and plenty of room laterally. It is frequently found in animals, but is rare in plants except in pollen-grains (Darlington, 1936, *a*).

In the pollen-tube (x P.T.) we find an entirely different arrangement. In the same organism the same number of chromosomes are present, but the shape of the cell is the very opposite of that found in pollen grains. There is no lateral freedom of movement, but an almost indefinite extension can take place along the length of the pollen tube. The spindle must therefore be long and narrow. In connexion with this another point may be mentioned. The nuclear membrane breaks down earlier in prophase than is usual, but owing to the elongated shape of the nucleus at this stage, and the difficulties of congression on the somewhat crowded plate, a greater length of time is required for this process and the chromosomes are normally contracted when the plate is formed. Furthermore, since the spindle probably determines the breakdown of the nuclear membrane, it must have been longer in developing than usual before congression is complete. It would appear from meiosis, in which there is non-pairing, that when congression does not occur the spindle elongates abnormally instead of expanding laterally. In the pollen-tube a similar elongation seems to occur normally and to be adapted to the peculiar requirements of the cell. The repulsion from the poles is not so great as in the pollen-grain, but, owing to the crowding, the mutual repulsions of the chromosomes and the centromeres is probably greater. The balance of these forces is altered, so that the centromere and body repulsions have a predominant role in the arrangement of the chromosomes. As a result, the chromosomes lie at right-angles to the plate, the centromeres being the only part of them actually on the plate. These conditions are modified slightly by variations in the width of the tube, or generative cell, and by the presence, in polyploid species, of different numbers of chromosomes, causing the plate to be displaced obliquely, as we have seen, or in extreme cases to be buckled (fig. 27, $2x$ P.T.).

In the root-tip ($2x$ R.T.) we have a condition intermediate between these two extremes. To begin with, there are in a given organism twice as many chromosomes present as in the preceding cases. The cell is usually much smaller than the pollen-grain, and at the most active part of the young root-tip approximately spherical. The poles may therefore be as close together as in the pollen-grain, with no space for lateral expansion, or

alternatively the width of the metaphase plate may be as small as in the pollen-tube, with no space for longitudinal expansion. In a root-tip, therefore (fig. 24, Plate 5), neither the centrosome nor the chromosome repulsion is free to dominate the arrangement of the chromosomes. They lie with their centromeres spread out on the plate as far as space will allow, but if the chromosomes are long, only a short portion of the two arms is actually on the plate. The distal ends are pushed off by their own body repulsions. An exactly similar arrangement occurs when the generative cell divides within the pollen-grain. Further back in the root-tip where the cells are already beginning to elongate, conditions become very similar to those found in the pollen-tube, and the plate is often similarly displaced.

I conclude, therefore, that the amount of space available and the size and number of the chromosomes present determine their arrangement at metaphase of mitosis, by determining the predominance of the centrosome repulsion, as in a pollen-grain, the predominance of the body-repulsion, as in the pollen-tube, or their approximately equal balance as in the root-tip.

SUMMARY

Mitosis in the generative cell of the pollen-tube differs from that in other parts of the plant in two main respects :—

- (1) In comparison with most dividing cells the pollen-tube is extremely narrow, but of almost unlimited length.
- (2) The nuclear membrane breaks down early in relation to the contraction of the chromosomes and the formation of the spindle, and owing to difficulties of movement the chromosomes are slow in coming on to the metaphase plate.

Both these factors determine a long, narrow spindle.

Comparison of mitosis in root-tips, pollen-grains and pollen-tubes shows that the arrangement of chromosomes on the metaphase plate is determined by the shape of the spindle, which is itself limited by the space available in the cell.

PLATE

(All photographs are from acetocarmine preparations, unless otherwise stated.)

FIGS. 19-22—*T. montana* ($\times$ ca. 500). Cf. figs. 1-4. Fig. 19—Prophase. The generative cell passing into the pollen-tube. Two hours after sowing. Fig. 20—Metaphase. The width of the plate is limited by the generative cell, not the pollen-tube. Fig. 21—Anaphase. FIG. 22—Telophase. A transverse cell-plate has been formed. The metaphase spirals are becoming uncoiled.

FIG. 23—Metaphase in the pollen-grain of *T. Clusiana*. All the chromosomes, except those in the centre, lie flat on the equatorial plate (cf. fig. 27). [$\times$ ca. 1000. 2BE and Gentian Violet.]

FIG. 24—Metaphase in the root-tip of *T. Gesneriana* var. Mrs. Moon. Those parts of the chromosomes adjacent to the centromere lie on the plate, while the ends are turned up or down (cf. fig. 27). [$\times$ ca. 1000. 2 BE and Gentian Violet.]

FIG. 25—Metaphase in the pollen-tube of *T. Greigi*, showing spiral structure (cf. fig. 16). The only part of the chromosome to lie on the plate is the centromere (cf. fig. 27). [$\times$ ca. 2000.]

FIG. 26—Telophase in the pollen-tube of *T. montana*, showing the metaphase spirals uncoiling (cf. fig. 18). [$\times$ ca. 2000.]

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Proteolytic Digestion and the Problem of the Pancreas in the Ammocoete Larva of *Lampetra planeri*

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[PLATE 6]

1—INTRODUCTION

Remarkably little experimental work has been carried out upon the digestive system of the Cyclostomata. Alcock (1899) described the occurrence of peptic digestion in the ammocoete, but in view of the early date of this work and of the fact that the protease of the Tunicata is now known to be of the tryptic rather than the peptic type (Yonge, 1925; Berrill, 1929) the matter seems to demand reinvestigation. Moreover, recent histological studies on the "pancreas" and intestine of lampreys, to be described below, have led to conclusions regarding the distribution of the zymogen cells which have not yet been tested by experimental methods. The present work deals with these two problems, to the significance of which attention has already been drawn elsewhere (Barrington, 1935), and provides also a basis for a comparison of proteolytic digestion in the Cyclostomata with that in the Tunicata and in the Pisces.

The ammocoete larvae of *Lampetra planeri* used in this work have been collected from the River Itchen in Hampshire at various times between April and September. Material for sectioning has been fixed in Bouin's solution, Lane's fixative, mercuric formol, and in Susa's solution, and subsequently stained on the slide with Mallory's stain, Heidenhain's haematoxylin, and by Lane's method for zymogen granules. The experimental methods will be described later.

2—MORPHOLOGICAL

The histology of the intestine of the ammocoete has recently been described and discussed by Maskell (1930), while the structure and development of the so-called "pancreas" has been intensively studied by various authors (Boenig, 1929; Cotronei, 1927; Keibel, 1927; etc.). No attempt will be made here to summarize all the details of this literature, but certain points directly relevant to the present work must be recapitulated.

Behind the pharynx the alimentary canal continues above the liver as a short and slender fore-gut or oesophagus (fig. 1, *oes*) which soon passes into the wide mid-gut or intestine (*ai*), this continuing straight back to the cloacal opening. The oesophagus, which is lined by a simple columnar epithelium, passes into the intestine somewhat ventrally (fig. 1) and gives the impression of being slightly invaginated into the latter (Picqué, 1913), a tip of the intestine, in other words, projecting forwards a little on each side of the oesophagus. The relations of these parts are illustrated in fig. 5, Plate 6, which is of a transverse section approximately through a plane *t* in fig. 1; in the mid-ventral line is seen the extreme hind end of the

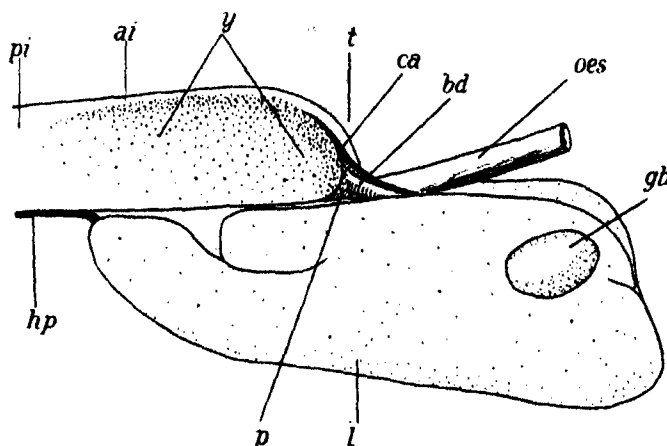


FIG. 1.—Right lateral view of the alimentary canal of the ammocoete in the region of the liver. *ai*, anterior intestine; *bd*, bile duct; *ca*, coeliac artery; *gb*, gall bladder; *hp*, hepatic portal vein; *l*, liver; *oes*, oesophagus; *p*, position of "pancreas"; *pi*, posterior intestine; *t*, approximate plane of the section shown in fig. 5, Plate 6; *y*, yellowish-white area.

oesophagus (*oes*) which passes on the left into the ventral region of the intestine (*ai*, fig. 5), while on the right a more dorsal region of the latter projects forwards as a mass of darkly staining cells (*cn*, fig. 5). The "pancreas" of the ammocoete develops at this point of junction as follicles (*f*, fig. 5) budded off from the gut epithelium into a surrounding mass of subepithelial tissue, and so comes to lie in the furrow formed (fig. 1, *p*) between the oesophagus, and the projecting tips of the intestine (Boenig, 1929). It is now established that these follicles lose their connexion with the gut epithelium, and Cotronei (1927) has advanced strong arguments, on histological grounds, that they constitute an exclusively endocrine or insular organ. Several authors (*e.g.*, Keibel, 1927) have described the follicles as being composed of light and dark cells, and

Cotronei believes that these are identical with the characteristic α and β cells of islet tissue. A further development of "pancreatic" tissue occurs at metamorphosis, but this need not be considered here.

Maskell (1930, 1932) has shown that at the corresponding point in the Australian ammocoete, *Geotria australis*, the intestine grows forward as a pair of blind diverticula lying one on either side of the oesophagus, the left one being much longer than the right, while in *Mordacia* (another Australian form) a similar diverticulum is present on the left side only. The resemblance between the diverticula of the Australian forms and the anterior end of the intestine in *Lampetra* is a close one, for the bile duct (fig. 1, *bd*) enters at the anterior end of the left diverticulum in the former, and at the anterior end of the intestine in the latter, while the "pancreatic" follicles develop in the Australian forms in the furrow between the diverticula and the oesophagus. The importance of these points in the present connexion lies in the peculiar histological structure of the regions of the intestine concerned.

The anterior end of the intestine in *Lampetra* is marked externally by two prominent yellowish-white areas (fig. 1, *y*) sharply separated from each other in the mid-dorsal line but with ill-defined ventral and posterior boundaries. The region of the intestinal epithelium corresponding to these areas is seen in transverse section, fig. 3, Plate 6, to differ from the epithelium immediately posterior to these areas (fig. 4, Plate 6) in containing cells (*cn*) which are more elongated and stain more darkly than the ordinary epithelial cells, and which have already been noted in fig. 5, Plate 5. Closer examination shows that these cells (fig. 6, Plate 5) are characterized by their granular protoplasm and by the possession of a vesicular nucleus with an exceptionally large nucleolus (*n*), while the ordinary epithelial cells (fig. 7, Plate 5) have a relatively clear protoplasm and a nucleus (*n*) with usually two much smaller nucleoli. Figs. 6 and 7, which illustrate these two types of cell, represent approximately corresponding points of the lateral wall of the two regions of the intestine seen in figs. 3 and 4 respectively, and have been photographed at the same magnification. These cells with large nucleoli are especially concentrated in the dorso-lateral wall of the intestine and of the spiral valve (fig. 3, *cn*, Plate 5), but they are also scattered in the more ventral regions of the epithelium. Their presence at the anterior end of the intestine has been noted by Brachet (1897), who suggested that they were an accumulation of zymogen cells, and represented an evolutionary stage in which the vertebrate pancreas had not yet separated from the intestinal epithelium, and by Picqué (1913), who believed that they gave rise to the "pancreatic" follicles. The latter suggestion has since been disproved

(Boenig, 1929), but the former has received support from Maskell (1930), who has described similar cells lining the diverticula of *Geotria*. He finds, and the present writer can confirm this of the corresponding cells in *Lampetra*, that the granules resemble zymogen granules in being stained purple by Lane's technique, while he points out that in the possession of a single large nucleolus (or rarely two) they agree with the zymogen cells of the pancreas of the trout, the frog, and the rat. The presence of these cells makes a sharp distinction between this region and both the oesophagus and the remainder of the intestine in *Lampetra*, and the two regions of the latter will therefore be referred to here for convenience as the anterior and posterior intestine, although it must be understood that the former is very much shorter than the latter. To take a particular example, in an intestine with a total length of 42 mm., the anterior region extended over only about 3.5 mm.

It remains to add that amongst the columnar cells lining the posterior intestine (fig. 4, Plate 5) are to be seen groups of cells which stain more darkly than their neighbours with haematoxylin. It seems likely that these also are secretory cells, but they are quite distinct from the specialized cells of the anterior end, their nucleoli being of the normal size. It is not in any case suggested that zymogen cells are to be found only in the anterior intestine, the essential fact for the present work being the localization in this region of a particular type of cell not found elsewhere in the epithelium.

3—EXPERIMENTAL

The p_H of the intestinal contents was measured by means of the B.D.H. Capillator, using bromo-thymol-blue and phenol-red as indicators, a small portion of the contents being removed from a freshly caught and decapitated animal and diluted with one drop of distilled water. By this method the p_H was found to range from 6.8 to 7.2 with bromo-thymol-blue, and from 7 to 7.4 with phenol-red. This discrepancy between the two indicators is probably due to their buffering action, which is particularly pronounced with the capillator method on account of the relatively high proportion of indicator which is used. If the results are expressed as the average of the two sets of readings it can be stated that the p_H range within the intestine is from about 6.9 to 7.3. No constant difference was found between the two ends of the intestine. Gunzberg's test for mineral acid gave negative results, as might be expected. It may therefore be concluded that the intestine does not provide a suitable environment for the action of pepsin.

Separate extracts were made of the gill epithelium, the liver, the oeso-

phagus, the anterior and posterior intestine, and the contents of the intestine; the tissues were always rinsed in distilled water, the intestine being first slit open and the contents removed. The various parts were ground up in water with silver-sand, left to stand for about 24 hours with a few drops of toluene added, and then diluted to make aqueous extracts of definite concentration—usually 1 gm. of tissue to 50 cc. of water (2% extract) or to 12.5 cc. of water (8% extract). Since the weights of tissue were always small, these concentrations could not be more than approximate, but they afford some basis for a comparison of the activity of the various extracts. The concentrations of the extracts are stated as 2% or 8% in the following tables.

Preliminary experiments were carried out with gelatine and fibrin as substrates. Following the method described by Dorris (1935), one drop of the extract was added to one drop of each of the appropriate buffers distributed in the compartments of a colour-palette, and one drop of each of the mixtures transferred to a photographic plate from which the silver salts had previously been dissolved out by hypo. This plate was allowed to stand for 2 hours at 24° C., and then washed and stained with acid fuchsin. Those drops in which proteolytic activity occurs leave a gelatine-free area on the plate which appears clear against the red background of the stained gelatine. Where digestion occurs in several drops it is often possible to form an estimate of their relative activity by comparing the areas of the gelatine-free spaces within the areas occupied by the original drops. Careful controls are necessary, as the alkaline buffers by themselves produce an effect which may be confused with that produced by a weak enzyme. Results are given in Table I.

TABLE I—SUBSTRATE: GELATINE FILM

<i>pH</i>	1.4	3	5	6	7	8
Anterior intestine, 2 % ..	—	—	+	++	+++	+++
Anterior intestine without “pancreas”, 2 %	—	—	+	++	+++	+++
Posterior intestine, 2%	—	—	—	—	—	—
Liver, 2%	—	—	—	—	—	—
Oesophagus, 2%	—	—	—	—	—	—
Controls	—	—	—	—	—	—

In experiments involving fibrin the congo-red fibrin of Roaf (1909) was used. Small tubes were prepared containing 0.05 gm. of the fibrin, 0.25 cc. of active or boiled extract, 0.75 cc. of buffer solution, and one drop of toluene, and were incubated at 37° C. for 48 hours and longer.

Digestive activity was estimated by the degree of staining of the fluid. Results are given in Table II.

TABLE II—SUBSTRATE: CONGO-RED FIBRIN

p_H	1.4	1.4	6	6	8	8
	active	control	active	control	active	control
Anterior intestine, 2%	none	none	slight	none	moderate	none
Posterior intestine, 2%	none	none	none	none	none	none
Liver, 2%	none	none	none	none	none	none

These experiments thus combine to show that proteolytic action occurs in extracts of the anterior end of the intestine, and that it is of the tryptic type. They were not carried any further as it was felt that the formol-caustic soda titration method, with casein as substrate, would provide a more satisfactory quantitative expression of the situation, and an extensive series of experiments using this method have therefore been carried out.

The technique adopted follows that suggested by Bayliss (1935). Tubes were prepared containing 0.5 cc. of active or boiled extract, 2 cc. of 2% casein, 1 cc. of buffer, and one drop of toluene, an active mixture and a boiled control being prepared for each p_H value. These pairs of tubes were left at 37° C. for 48 hours, and at the end of this time the two tubes of each pair were titrated against each other. 1 cc. was removed from each, one drop of phenol-phthalein added, and the two titrated to the same pink colour with N/50 caustic soda; 1 cc. of neutral formalin was then added, and the two again titrated to the same colour. The difference between these last two titration figures is, of course, a measure of the amino-acid content of the active mixture, and hence of its proteolytic activity. The p_H of each tube was measured before and after incubation, using the capillator, and the actual p_H was taken to be the average of the two readings.

The results, as shown in fig. 2, confirm the preliminary experiments in showing that the anterior intestine produces an enzyme of the tryptic type. Three consecutive series of experiments gave the point of optimum activity as p_H 7.5, 7.8, and 7.5, and the optimum may be considered as lying between these points. The essential fact demonstrated by the readings for the posterior intestine and the liver (fig. 2) is that from the point of view of digestion their proteolytic activity is negligible. As they stand, these readings show no more than this, for the differences between their figures are too small to be significant; but several series of titrations carried out with 2% and also with 8% extracts have shown very con-

sistently that both liver and posterior intestine have a slight proteolytic activity in an acid medium, even with HCl present (Clarke and Lubbs chloride buffer), and that the intestine has also a slight activity in an alkaline medium, the liver probably not sharing this property (Table III).

TABLE III—SUBSTRATE: CASEIN. AMINO-ACID EQUIVALENT OF ACTIVE DIGEST IN CC. OF N/50 CAUSTIC SODA PER 1 CC.

Posterior intestine, 8%	p_H	4.5	6.4	8.1
	cc. of NaOH	0.07	0.05	0.04
Liver, 8%	p_H	4.5	6.4	8.1
	cc. of NaOH	0.13	0.12	0

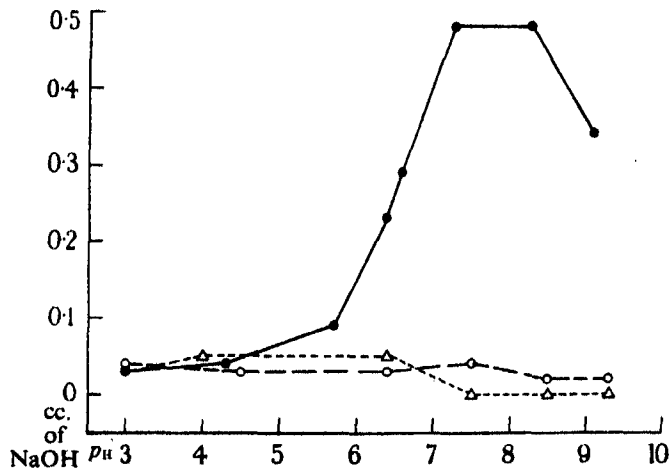


FIG. 2—Proteolytic activity, by formol-caustic soda titration, of 2% extracts of anterior intestine ●; posterior intestine ○; and liver Δ. Substrate: casein. Ordinates: amino-acid equivalent of active digest in cc. of N/50 caustic soda per cc.

It will be noted that the activity of the anterior intestine also extends into a region of considerable acidity (fig. 2). It would seem reasonable to suppose that this activity of all the extracts in acid media results from the presence of an autolytic enzyme (cathepsin) which would be expected to be operative over such a p_H range. The slight activity of the posterior intestine in alkaline media may be due either to a slight production of the enzyme throughout the length of the intestine, or to a failure to remove all traces of the intestinal contents, but in any case this activity is too weak to be of significance in digestion. Thus these results shed no further light upon the nature of the presumed secretory cells found in the posterior intestine, for the latter are far more numerous than would be necessary to account for the production of only a trace of protease. It

may well be, however, that other enzymes are less localized in their distribution than is the latter, and unpublished work of the author suggests that this is true of at least the amylase.

In most of the experiments carried out upon the anterior intestine the "pancreas" has been included in the tissues extracted. In order, however, to guard against the possibility that the proteolytic activity of these extracts might be due to these follicles, a few experiments have been performed with extracts from which they had been entirely excluded by cutting off the tip of the intestine with which, as has been explained, they are in intimate contact. Such extracts (Tables I and IV) still exhibited proteolytic activity, from which it may be concluded that the presence of the "pancreas" is not necessary for proteolytic action, a conclusion which is consonant with the work of Cotronei referred to above.

TABLE IV—*Cf.* TABLE III

Anterior intestine without "pancreas", 2%..	PH.....	7.3	7.8
	cc. of NaOH	0.44	0.51

The oesophagus is too small to allow of any extensive experiments being carried out upon it, but such as have been performed (Table V) indicate that proteolytic activity is absent or negligible.

TABLE V—*Cf.* TABLE III

Oesophagus, 2%	PH.....	8.4
	cc. of NaOH	0

In view of Miss Alcock's statement that the pharynx is the region of the alimentary canal most active in proteolytic digestion, extracts of the gill epithelium have been tested. The results (Table VI) show that the activity of this region is of the same negligible order as that of the posterior intestine. This, as will be explained below, is not so direct a contradiction of her conclusion as it appears to be, for she was misled throughout her work by her failure to discover the tryptic enzyme of the intestine.

TABLE VI—*Cf.* TABLE III

Gill epithelium, 2%	PH.....	4.6	6.3	7.5	9.3
	cc. of NaOH	0.02	0.01	0.03	—0.01

4—DISCUSSION

From the results described above, two main conclusions are to be drawn—that proteolytic digestion in the ammocoete is of the tryptic

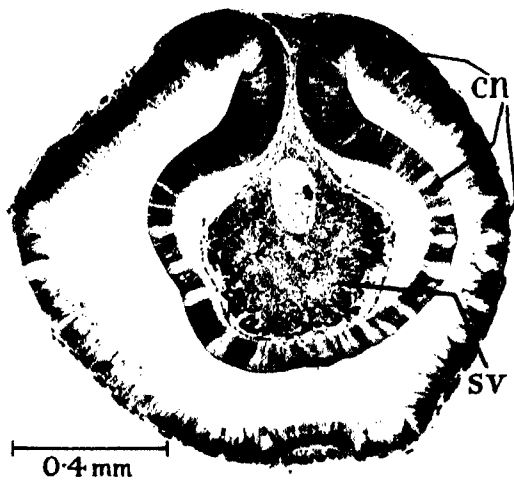


FIG. 3.

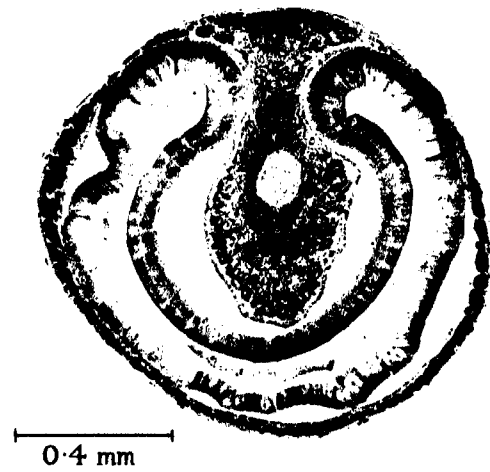


FIG. 4.

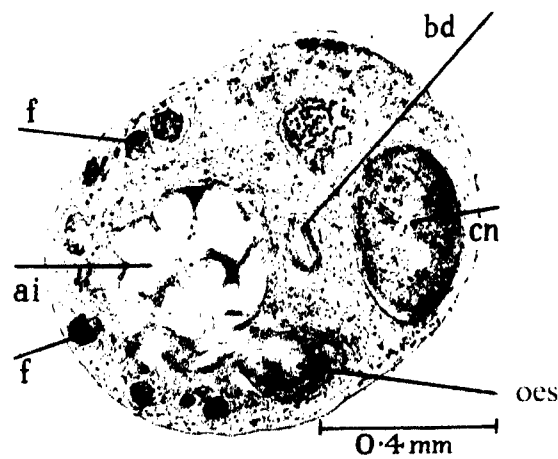


FIG. 5.

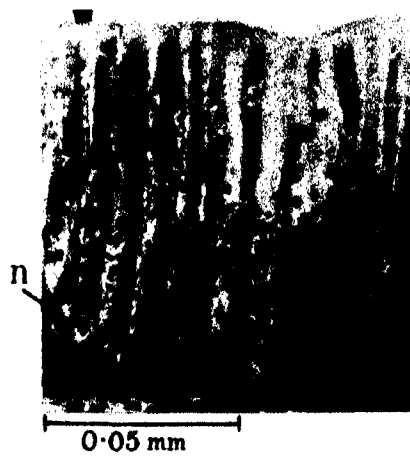


FIG. 6.

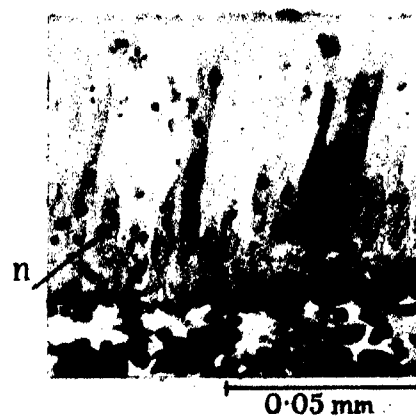


FIG. 7.

type, with an optimum activity between p_H 7.5 and 7.8, and that the production of the enzyme concerned is localized at the anterior end of the intestine. With regard to the nature of the protease, there seems to be a close resemblance between the ammocoete and the Tunicates. Yonge (1925) described in *Ascidia* a weak protease active in alkaline and neutral media, and later Berrill (1929) showed that in *Ciona* the protease was active from p_H 6 to above 10, and that the p_H of the intestine ranged from 6.8 to 7.4, a very close agreement with the conditions in the ammocoete. Using a method involving the liquefaction of gelatine, he finds the optimum for the enzyme to be at about p_H 8, a figure only slightly higher than that recorded here for the latter animal. Although in both of these forms the optimum for the enzyme is slightly above the p_H range within the intestine, it is apparent from fig. 2 that in the ammocoete, at least, the protease is well adapted for functioning in the environment provided, for even at the lower p_H limit of 6.9 there is an ample margin of proteolytic activity.

Bayliss (1935) has recently published an account of digestion in the Plaice (*Pleuronectes platessa*), and since he has studied the proteolytic activity by the formol-titration method it is possible to make a direct comparison between the conditions obtaining in that animal and in the ammocoete. The p_H of the intestine of the Plaice is found to range from 7.43 to 8.65, and there is (in addition to the pepsin of the stomach) a trypsin with an optimum between 7.5 and 8.5. Whether the difference between the trypsin of this animal and that of the ammocoete is significant it is impossible to say, but the increased alkalinity in the intestine of the former certainly provides a better correlation between enzyme and environment than obtains in the latter. Such a difference is perhaps an example, although a slight one, of the improvement in the organization of the digestive system which has marked the transition from the Cyclostomata to the Pisces. It is of interest that Bayliss finds a slight activity in acid media, and ascribes this to the presence of a cathepsin, as has been suggested above for the ammocoete.

It has been mentioned that Miss Alcock (1899) described proteolytic digestion in the ammocoete as being of the peptic type, and since such a conclusion is directly controverted by the results recorded here, it is natural to ask how such a contradiction could have arisen. It must be realized, first of all, that her work was carried out at a time when the importance of p_H control was still unrecognized, the acidity of the media in her experiments being secured merely by the presence of 0.2% HCl; her results, based upon the digestion of carmine-stained fibrin, thus show nothing more than that some digestion occurs in the presence of HCl, and

offer no definite evidence as to the nature of the enzyme involved. Since, however, the present work has also shown that some digestion does occur in the presence of HCl, it is clear that the disagreement is one of interpretation rather than of fact. Indeed, there is an agreement in at least one respect, for she writes that "as far as the alimentary canal is concerned, the liver is the most active organ, then comes the pharynx, and lastly the intestine". Table III shows that the formol-titration method also reveals the liver as being more active than the intestine in acid media, although no significant difference is revealed between the pharynx and the intestine. That Miss Alcock was dealing with a very weak enzyme is suggested by her statement that tissues proved "inefficient" when they had been dissected under water, or had been obtained from animals which had been kept for some months in the laboratory, and it must be concluded, as has been suggested above, that she was actually investigating a weak cathepsin.

Her failure to detect any proteolytic activity in alkaline media may be ascribed partly to her method, which involved adding to the extract 1% sodium carbonate and unstained fibrin, but mainly to the fact that she made extracts of the whole length of the intestine, in which the intense activity of the anterior end would inevitably be masked by the inactivity of the remainder of it. It is significant that she failed to find any trace of a diastatic ferment, although the present writer has found no difficulty in this respect (unpublished results).

It has been shown that the region of production of the protease corresponds exactly with the area occupied by the granular cells with prominent nucleoli, a fact which provides physiological proof of the suggestion of Brachet and Maskell that these are zymogen cells, and is in agreement with the conclusion of Cotronei that the "pancreas" is exclusively endocrine, for the proteolytic activity proceeds in the absence of this organ. It thus appears that in the evolution of the pancreas in the Chordata the endocrine portion separated from the gut epithelium and became compacted into a definite organ while the exocrine portion was still confined to that epithelium. The ammocoete may be regarded as representing an intermediate stage in which zymogen cells of a special type are becoming localized preparatory to separating from the intestinal epithelium, and they are so close to the endocrine portion (fig. 5, Plate 6) that the transition to a true pancreas, composed of exocrine and endocrine portions, is not difficult to visualize. The formation at the anterior end of the intestine in *Geotria* and *Mordacia* of intestinal diverticula containing these zymogen cells gives some indication of how the further evolution might have proceeded; but there is no reason, of course, for expecting such a

specialized group as the Cyclostomata to provide an exact picture of the evolution of the organ. The complexity of this region, indeed, makes it very difficult, if not impossible, to identify in the ammocoete the dorsal and ventral pancreas characteristic of the developmental stages of the higher Chordata. It is true that two main portions (anterior and posterior) are to be distinguished in the "insular organ" or "pancreas" of the adult lamprey, and Boenig (1929) has regarded these as representing the dorsal and ventral pancreas respectively, but it is clear that in view of the fact, demonstrated here physiologically for the first time, that the zymogen cells are still located in the intestine, any such comparison must necessarily be incomplete.

The writer is indebted to Professor E. S. Goodrich and Dr. G. R. de Beer for reading this work in manuscript, to Professor C. M. Yonge and Mr. J. Z. Young for advice on certain matters, and to Mr. F. T. K. Pentelow for the facilities enjoyed during several visits to the Fisheries Research Station, Alresford, Hants.

5—CONCLUSIONS

The ammocoete possesses a proteolytic enzyme of the tryptic type, with an optimum between p_H 7.5 and 7.8.

The production of this enzyme is localized at the anterior end of the intestine, in a region where a concentration of presumed zymogen cells of a specialized type had been described by previous workers.

The presence of the "pancreas" ("insular organ" of Cotronei) is not essential for the production of the enzyme.

The results indicate a closer physiological resemblance between the process of proteolytic digestion in the Tunicata and the Cyclostomata than had previously been recognized, and throw some light on the evolution of the pancreas in the vertebrates.

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DESCRIPTION OF PLATE 6

- FIG. 3—Transverse section of the anterior intestine of an ammocoete.
 FIG. 4—Transverse section of the posterior intestine.
 FIG. 5—Transverse section of the alimentary canal at the point of junction of oesophagus and intestine.
 FIG. 6—Presumed zymogen cells with large nucleoli in the epithelium of the anterior intestine.
 FIG. 7—Ordinary columnar cells in the epithelium of the posterior intestine.
Explanation of lettering—*bi*, bile duct; *ai*, anterior intestine; *ca*, coeliac artery; *cn*, cells with large nucleoli; *f*, follicles of "pancreas"; *n*, nucleus; *oes*, oesophagus; *sv*, spiral valve of intestine.

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Royal Society Expedition to Montserrat, B.W.I.: Preliminary Report on the Geology of Montserrat

By A. G. MACGREGOR, F.R.S.E., F.G.S.

(Communicated by Sir John Flett, F.R.S.—Received 18 July, 1936)

[PLATES 7-9]

INTRODUCTORY

This preliminary account of the geology of Montserrat is based on field work carried out between 9 March and 6 May, 1936, and later between 14 May and 3 June. During a visit paid to Martinique (8 to 13 May) three and a half days were devoted to a study of Mt. Pelé and its surroundings. In the course of the voyages between Barbados and Montserrat on my outward and homeward passages, I saw from the sea the islands of St. Lucia, Dominica, The Saints, Marie Galante, and Guadeloupe, and during short motor trips in Barbados, St. Lucia, and Dominica gained a further idea of the main topographical features of these islands. While travelling by air from Martinique to Antigua, I had good bird's eye views of the west and north of Mt. Pelé, of the east

coast of Dominica, and of Guadeloupe and Antigua. Redonda and Nevis were well seen on clear days from the northern end of Montserrat.

To His Excellency Sir Gordon Letham, Governor of the Leeward Islands, and to the Commissioner and acting Commissioners of Montserrat, the Royal Society Expedition is indebted for much kindness, advice, and assistance. I am also most grateful to officials of the Montserrat Department of Agriculture, of the Montserrat Company, and of the local Police Force, as well as to all estate owners and managers, for guidance and information and for the frequent loan of horses and men. Finally, I am greatly indebted to Mr. T. Savage English for placing his extensive local knowledge at my disposal.

In Martinique, Mr. F. A. Perret gave me, in field and museum, the benefit of his intimate knowledge of Mt. Pelé, while Mr. Meagher, the British Consul at Fort de France, met me on my arrival and helped me in every conceivable way. I am greatly indebted to them for the trouble they took to make my short visit a success.

PREVIOUS GEOLOGICAL WORK

Little systematic geological field work has been undertaken previously in Montserrat. Karl Sapper (1903) a German volcanologist, spent a week in the island in 1903 and published some notes. Hardy (1932), Chemist to the Imperial Department of Agriculture for the West Indies, after a short visit in 1921, wrote a paper on the soils, with notes on climatic conditions. Dr. K. W. Earle, while Government Geologist to the Windward and Leeward Islands, spent a short time in Montserrat and wrote a brief account of the physiography and geology. He very kindly provided me with a typescript copy of his unpublished report (1923). Davis (1926), the American physiographer, while passing in a steamer, fitted the island into his physiographic evolution scheme for the Lesser Antilles. Mr. F. A. Perret, an American volcanologist, visited the island several times during the 1933-6 series of earthquakes and presented to the Governor of the Leeward Islands a number of reports on earthquakes and *soufrières* which were printed by the Colonial Office. Mr. T. Savage English kindly lent me the manuscript of his "*Records of Montserrat*", which contains some geological notes, and Mr. C. A. Gomez, Curator of the Botanical Station, placed at my disposal an account of the earthquake and *soufrière* activity which he had prepared officially for the Government.

Sapper (1903) regarded the main mountain masses as denuded volcanoes, denied that Chance's Pond is a crater lake, described Gage's

Lower Soufrière and Galway's Soufrière, collected fossils from the tuffs at Sweeny's Bay, and recognized lavas between Sweeny's Bay and O'Garra's. Earle observed that the tuffs on the whole dip away from the main and subsidiary mountain masses, gave a brief petrographical description of one or two specimens of andesite, collected interesting minerals from two soufrières, and fossils from Sweeny's Bay (Landing Bay). The fossils he regards as possibly of Pleistocene Age. English was the first to claim the presence of an old crater, with a central mass of igneous rock, in the Tar River area. He described Cow Hill Soufrière, Hot Water Pond, the local black beach sands, etc.

THE VOLCANIC ARC OF THE LESSER ANTILLES

Montserrat (lat. $16^{\circ} 45' N.$, long. $62^{\circ} 10' W.$) in the Leeward Islands, a roughly pear-shaped mountainous volcanic mass about 32 square miles in area, is a small member of the western series of islands that form the volcanic arc of the Lesser Antilles or Caribbees. The islands from north to south are Saba, St. Eustatius (Statia), St. Kitts, Nevis, Redonda, Montserrat, Basse Terre (western part of Guadeloupe), Dominica, Martinique, St. Lucia, St. Vincent, The Grenadines, and Grenada. Intermittent volcanic activity has been going on along this arc since at least Miocene times, about 20 to 25 million years ago. Most of the islands still have mineral or hot springs and active soufrières, while a number contain well-preserved volcanic cones with craters. Eruptions have taken place within historic times in St. Kitts, Guadeloupe, Dominica, Martinique, St. Lucia, and St. Vincent. Much the most serious were the great eruptions of Mt. Pelé in Martinique and of the Soufrière of St. Vincent in 1902. Another serious outbreak at Mt. Pelé began in 1929 and did not end till 1932. At various times in the past, earthquake epicentres have been located in all the larger islands, except apparently St. Lucia.

In Montserrat there have been no eruptions in historic times, but from 1897 until the outbreak of Mt. Pelé and the Soufrière of St. Vincent in 1902, the activity of the soufrières showed a marked increase and numerous local earthquakes occurred. Later, in 1933, began another series of earthquakes and a renewal of abnormal soufrière activity, which have not yet died away.

Martinique, with the dormant Mt. Pelé volcano, is situated about the middle of the volcanic arc, which is convex eastwards. From Mt. Pelé Montserrat lies about 155 miles north-westwards along the arc, while St. Vincent is 125 miles southwards.

The Lesser Antilles include also an older, outer (north-eastern) belt comprising low-lying islands formed of volcanic rocks capped by Tertiary limestones of different ages. These "Limestone Caribbees" are Sombrero, Dog, Anguilla, St. Martin, St. Bartholomew, Barbuda, Antigua, Grande Terre of Guadeloupe, Désirade, Petite Terre, and Marie Galante. The old volcanoes that form their foundations had become extinct, and were eroded and more or less bevelled across before late Eocene times; they were then variously submerged and encrusted with Tertiary limestone and subsequently uplifted again.

TOPOGRAPHY OF MONTSEERRAT

The island of Montserrat, $10\frac{1}{2}$ miles long and 6 miles broad, is made up of six adjacent mountain masses and hills, each of which represents an old volcano modified by long continued erosion. From north to south they are: Silver Hill, Centre Hills, Garibaldi Hill, St. George's Hill, Soufrière Hills, and South Soufrière Hill (Roche's Mountain). Each of these units has given rise to its own system of radial drainage. They are generally separated from each other by well-marked hollows, or by zones of relatively low ground. These are original, not erosional or tectonic, features, although they have locally influenced the formation of stream courses.

The motor road that runs north-eastwards across the island from Plymouth, the capital (on the west coast), to Trant's (on the windward coast), more or less follows the mutual junction of the Soufrière Hills volcano with the St. George's Hill and Centre Hills volcanoes to the north. Other motoring roads, which run from Plymouth to O'Garra's on the south coast and to St. John's (via Cars Bay) in the northern part of the island, cut across the radial drainage channels, locally known as "ghauts" or "guts", for a large part of their extent. The most northerly part of the road system, however, follows, in the main, radial ridges of the Centre Hills. Communication north of St. John's and from St. John's south-eastwards to Trant's is by rough hill tracks. A motor road runs south from Trant's to Tar River, but from here round the south end of the island to O'Garra's, only a bad pony track is available.

The most up-to-date map obtainable is the hachured Admiralty Chart of 1869, on the scale of 1·72 inches to the land mile. On this the general topography of the island is well brought out, but main roads, tracks, and buildings are in many instances inaccurately located or unrecorded. The detail of the hachuring is also very often quite misleading. The

chart, however, is admirable considering its antiquity; it shows very clearly the radial drainage of the mountain and hill units, and brings out the fact that the valley systems of the Centre Hills and Silver Hill are much more mature than that of the Soufrière Hills. The map (fig. 1) is a modification of this chart.

A small map of Montserrat, with form-lines instead of hachures, appears on the United States Hydrographic Office Chart No. 1011. Here the scale is less than a third of that used for the British Admiralty Chart.

The water supply of the island is derived from mountain springs. Most of the ghauts are dry except in wet weather, but a few of the valleys contain permanent streams. The average annual rainfall in the less elevated regions varies in different parts of the island from about 41 to 78 inches, the leeward side of the island being wetter than the windward. The wettest months are generally September, October, and November.

In the Centre (2450 ft.),* Soufrière (3002 ft.) and South Soufrière (2505 ft.) Hills, the slopes above about 1000 to 1500 ft. are covered by dense tropical forest. The lower slopes, including often the precipitous sides of ghauts, are intensively cultivated. Silver Hill (1285 ft.) is now deforested to the summit. The chief crops are at present sea-island cotton, limes, and tomatoes.

RELATION OF PHYSICAL FEATURES TO GEOLOGY

It has already been pointed out that each of the old volcanoes gives rise to a hill or group of hills with radial drainage; one of the most striking scenic features of Montserrat (which is not brought out by the Admiralty Chart) is the abrupt rise of the steep forested slopes of the volcanic cores of the Soufrière and Centre Hills above the surrounding gently sloping cultivated land. To some extent this is due to the originally great height and steepness of the central portions or cores of the volcanoes. The main factors, however, are differences in structure and lithology. The central cores include a considerable amount of solid intrusive andesite,† and relatively hard, and probably unstratified or steeply dipping agglomerate, while the surrounding deposits usually consist of rather unconsolidated stratified series of agglomerates, tuffs, volcanic sands, etc., radiating outwards with a dip that does not as a rule exceed 10°. Differential erosion has, therefore, accentuated the relief of the more resistant

* Heights are taken from Admiralty Chart.

† Andesite is used in this preliminary report as a field term to cover all the andesitic looking rocks of Montserrat. Some of them may prove on microscopic examination to be dacite.

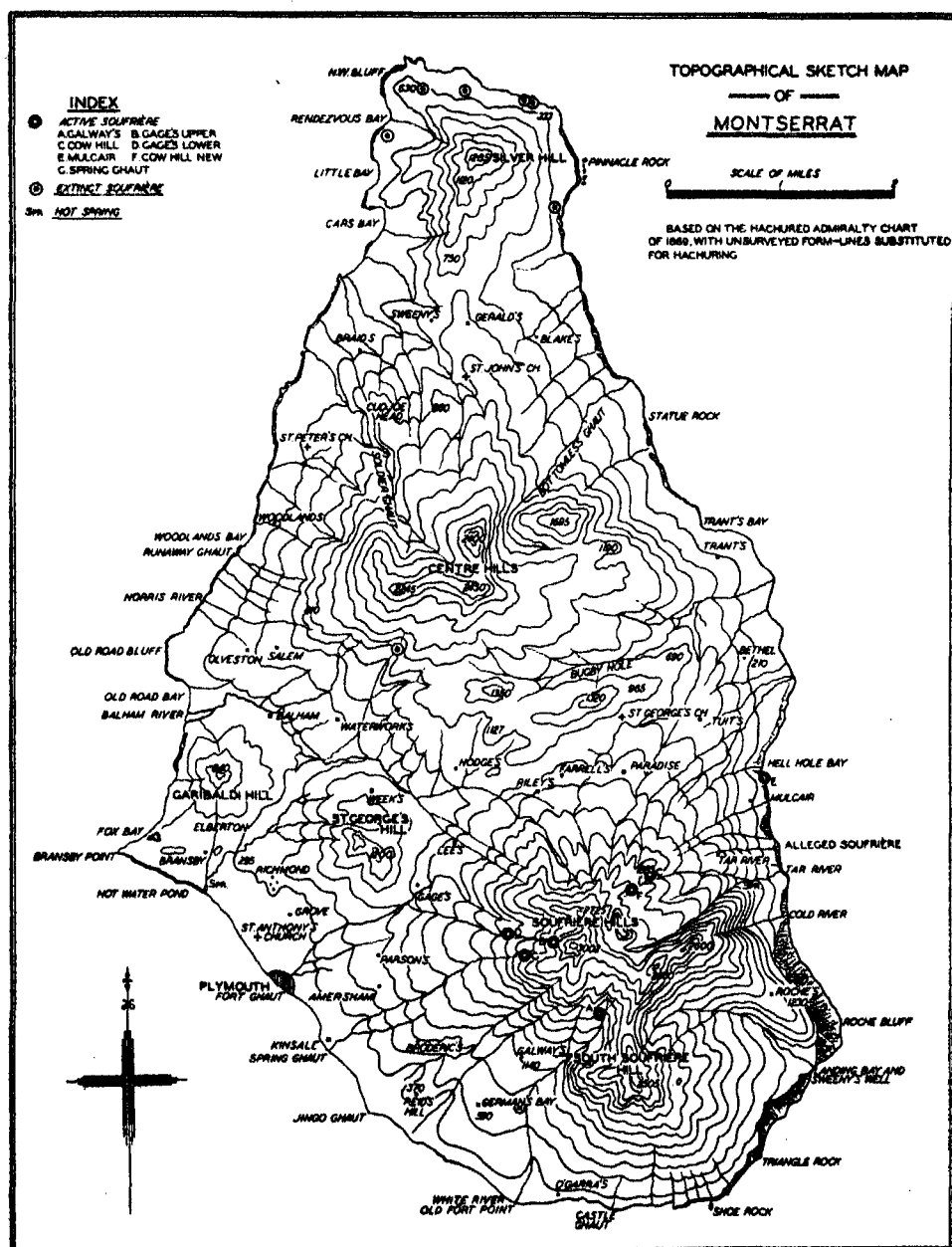


FIG. 1.

(Heights are shown in feet, e.g., 3002)

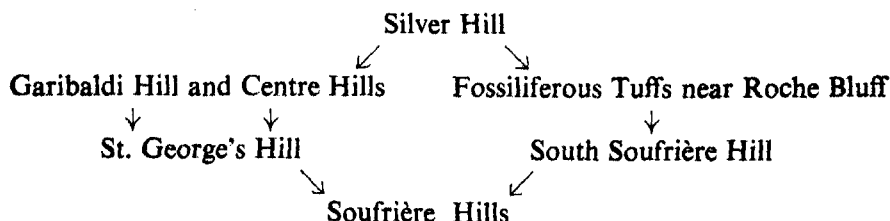
volcanic cores. Both parts of the old cones are, of course, deeply trenched by the radial ghauts, fig. 7, Plate 9.

The steep slope of the south-eastern flanks of South Soufrière Hill is due essentially to the original steep dip (25° – 30°) of the series of inter-bedded lava flows and tuffs which here form the hill. Undoubted lava flows are found only in the south-eastern corner of the island.

The major portion of the coastline is fringed by cliffs which, as Davis pointed out, characteristically truncate the spurs between the radial mountain valleys, as is also the case in most of the other islands of the Caribbean volcanic arc. Hanging valleys have been produced locally between truncated spurs. There are no raised beaches on the island, but locally there are low narrow platforms bordering the sea. These appear to be due to the accumulation of debris carried down the ghauts during periods of torrential rainfall.

SUMMARY OF VOLCANIC HISTORY

The oldest rocks in the island are probably those of Silver Hill in the extreme north. The sequence of eruptive activity, so far as is known, may be represented by the following diagram, in which the relative positions of names have no geographical significance:—



The arrows lead from any one volcano to a geographical neighbour of later date.

No direct field evidence was found which would indicate the relative ages of the volcanic centres of Silver Hill and Roche Bluff, or of the centres on the left of the page as compared with those on the right. The periods of eruption of South Soufrière Hill and of the Soufrière Hills probably overlapped to some extent, and the evidence that the Soufrière Hills are younger than St. George's Hill is not conclusive. An outline of the nature of the age evidence will be given in a later section.

Fossils collected by me from the Roche Bluff area were submitted to Dr. J. Pringle, the Survey Palaeontologist, who, in consultation with Dr. H. Dighton Thomas of the British Museum of Natural History and Mr.

A. G. Davis of the Anglo-Iranian Oil Company, has already identified many of them. Dr. Pringle reports that the collection includes algae, foraminifera, corals, spines of echinoderms, serpulæ, lamellibranchs and ostracods, and that the fauna and conditions of preservation suggest a Pleistocene age. This means that the eruptions of the South Soufrière Hill and Soufrière Hills volcanoes took place within the last 1 or 2 million years (and are therefore geologically very recent), for these volcanoes are demonstrably younger than the fossiliferous tuffs (pp. 241, 242).

Many Carib relics in the form of beads and pottery have been turned up close to the surface in the fields near Trant's. The fields are formed by some of the most recent volcanic products of the Soufrière Hills, though these have probably been covered to some extent by later alluvial outwash. If the age of the relics could be fixed by archeologists this, as Dr. Powell (Physicist to the Royal Society Expedition) suggested to me, would set an upper limit for the volcanic activity in Montserrat. Most of the relics are in the Museum of the America Indian (Heye Foundation) in New York, but a few are in the British Museum.

Capt. T. A. Joyce of the British Museum tells me, however, that he cannot assign any date to the relics. All that is known is that Caribs were in occupation of Montserrat when it was discovered by Columbus in 1493.

The active *soufrières* of the island are all situated in the Soufrière Hills area, where the most recent eruptions have occurred. As a result of plotting their positions on the map it has been found that a number of these lie approximately on a line passing across the summit area of the Soufrière Hills, in a direction about E. 35° N. They are Spring Ghaut Soufrière, Gage's Upper Soufrière, the Cow Hill (or Tar River) Soufrières, and an inaccessible *soufrière* on the east coast near Mulcair. Little or no definite evidence of faulting of the surface rocks has been found along this zone, but it may well represent a line of deep-seated crustal weakness. On another almost parallel line, about 1000 yards to the south-east, lie (1) an old locus of *soufrière* activity in White River Ghaut east of German's Bay; (2) Galway's (or Roche's) Soufrière; (3) hot springs near Tar River; (4) an alleged *soufrière* on the coast near Tar River.

One of the most important active *soufrières* (Gage's Lower), to which Mr. Perret has devoted so much attention, does not lie on either of these lines but is situated about 500 yards to the north-west of that first mentioned.

Hot springs that feed Hot Water Pond, on the west coast south of Garibaldi Hill, lie beyond the limit of the deposits of the Soufrière Hills volcano, and are a long way from all other hot springs and *soufrières*.

In the Silver Hill area, in the north of the island, evidence of former soufrière activity is abundant. In all, the sites of six old soufrières have been located approximately. Sites of old soufrières could doubtless be found in the Centre Hills area by careful search; only one or possibly two have been located.

The most recent event in the volcanic history of the island is the production of landslips and rock-falls by the more severe of the present series of earthquakes. They are very numerous on the precipitous wooded slopes of the central part of the Soufrière Hills, where they are conspicuous because the pale colours of the rocks laid bare show up against the surrounding dark green vegetation, fig. 8, Plate 9. The landslips and rock-falls are more numerous here than anywhere else in the island, though there are some on St. George's Hill and on the steep slopes of the Centre Hills and South Soufrière Hill, and much rock has fallen from the coastal cliffs, as for instance, between O'Garra's and Tar River. The slips and falls have affected both agglomerate and solid andesite, and many of the andesite blocks in steep "screes" are several feet in diameter. But for the exposures due to the earthquakes, practically nothing would be seen of the rocks of the core of the Soufrière Hills.

Numerous falls have also occurred along the motoring roads, which, from their construction, generally have a cliff of loose semi-consolidated agglomerate on at least one side. Slips from these cliffs occur frequently without the aid of earthquakes.

DESCRIPTION OF VOLCANIC CENTRES

Brief accounts will now be given of the more important features of each volcanic centre in turn. In order to describe most clearly the field evidence bearing on the relative ages of different centres it will be convenient to begin with the southern half of the island, including St. George's Hill. Garibaldi Hill, the Centre Hills, and Silver Hill, which together make up the northern half, will be dealt with later.

Roche Bluff Area—The oldest rocks of the southern part of Montserrat include the fossiliferous tuffs that occur in the coastal cliffs 300 yards north-east of Sweeny's Well (at Landing Bay), and they also extend along the east coast for 1500 yards north-north-westwards from Roche Bluff, fig. 6, Plate 8.

So far as can be gathered, Sapper and Earle saw only the restricted exposure close to Sweeny's Well. From the white marine limestone here incorporated in the tuffs, Earle records corals, lamellibranchs, calcareous

algae, Terebratulids, and spines and fragments of tests of echinoids. The writer found another good locality for fossils in the cliff about 1500 yards north-north-west of Roche Bluff (*cf.* p. 238).

Sapper's description of the mode of occurrence of these fossiliferous tuffs is somewhat misleading, but Earle's is correct so far as it goes. Although there are difficulties in deducing certain details of the volcanic history of the rocks exposed in the cliffs of the Roche Bluff area, it is clear that the pale bedded tuffs, in which the broken up masses of limestone occur, have been steeply tilted and locally brecciated on a large scale, and then covered unconformably by the more basic interbedded lava and tuff series, which occurs around Roche's and on the seaward slopes of South Soufrière Hill as far south-westwards as O'Garra's.

A little to the south-west of Sweeny's Well the coastal cliffs show the lava—tuff series of South Soufrière Hill resting unconformably on a very pale mass of andesite, which locally passes into breccia. This pale andesite is presumably of the same age as the pale fossiliferous tuffs to the north of Sweeny's Well; it does not extend more than about 1200 yards south-west of Landing Bay.

South Soufrière Hill—Interbedded lavas and tuffs form the eastern, south-eastern, and southern slopes of South Soufrière Hill. Most of the lavas are more basic looking than the igneous rocks in other parts of the island, and many are basaltic and olivine-bearing. Phenocrysts of feldspar and ferromagnesian minerals are in many instances small and inconspicuous. This is the only place where undoubted lava flows occur in Montserrat. They form numerous outcrops on the steep hill slopes, but it is impossible to tell how many successive flows are represented. South-east of the summit of the hill the dip is steep (25° to 30°), but the slope of the hillsides is usually somewhat steeper. The lavas are very poorly jointed and have slaggy, scoriaceous, and clinkery tops and bottoms; they vary from 12 ft. or less to about 40 ft. in thickness. The vesicles are empty and the lava tops show no trace of contemporaneous decomposition (formation of "bole"). The most westerly lava is finely exposed behind the buildings at the road end at O'Garra's.

The associated tuffs are largely composed of dark grey to black slaggy fragments and are often very evenly bedded. They are normal volcanic ashes and are easily distinguishable from any volcanic ejectamenta elsewhere on the island. The dark tuffs extend westwards of South Soufrière Hill, well beyond the limits of the lavas. Magnificent sections are seen in the high walls of White River Ghaut, which reaches the coast at Old Fort Point.

The dips of the lava—tuff and black slaggy tuff series are south-eastwards in the neighbourhood of Triangle Rock, due south 1000 yards west of Shoe Rock, south-west near the spur east of White River Ghaut, and approximately westwards in the neighbourhood of Galway's. This indicates an eruptive source somewhere near the summit of South Soufrière Hill.

In the ghaut and on the hill slopes just east of it, the black slaggy tuffs are interbedded with, and apparently capped by, bands of pale tuff, including pale pumice. West of the gaut, on the Galway's-German's Bay spur, the surface rocks belong to the *nuée ardente* type of andesitic agglomerate to be described later in connexion with the Soufrière Hills volcano. The most westerly exposures of the black slaggy tuff series occur in the bottom of Jingo Ghaut just north-east of Reid's Hill, where they have a westerly dip. Here they are overlain by a considerable thickness of typical Soufrière Hills agglomerate. It is thus clear that the eruptive products of the Soufrière Hills have covered up the black slaggy tuff series of an earlier South Soufrière Hill volcano. The interstratification of pale tuffs with the dark series in the neighbourhood of White River Ghaut suggests that the period of eruption of the two volcanoes overlapped to some extent.

Soufrière Hills—The general topographical and geological features of this volcanic centre have already been described. More detailed accounts will now be given of the deposits of the gently inclined outer slopes and of the rocks of the high central area, including "English's Crater".

The deposits of the outer slopes extend to the sea, on the west coast between Old Fort Point and Plymouth and on the east from Trant's Bay to the neighbourhood of Tar River. As a rule the surface of the ground is littered with andesite blocks of all sizes up to masses 12 ft. or more in diameter; their distribution is not uniform. The great majority of the blocks are porous* and banded and contain fine-grained grey igneous xenoliths. Many of the blocks, owing to their porosity, lend themselves readily to dressing by masons and have been used in the construction of local buildings, including churches and the old windmill towers.

The gently dipping deposits on which the surface blocks rest, and from which they have been derived, are almost everywhere practically unconsolidated. Different layers vary in character from confused aggregates of subangular andesite fragments with very little matrix, to beds

* Here the word "porous" is used to describe andesite intermediate in character between normal solid igneous rock and pumice.

of volcanic sand or dust. The most prevalent type consists of rock-fragments and blocks of varying size embedded in a friable matrix of pale grey volcanic sand and dust. Ill-defined bedding is usually determined by the alternation of beds in which either the average size of the stones, or the proportion of rock-fragments to matrix, is different, fig. 6, Plate 8. Layers of volcanic sand or dust, from a fraction of an inch to a foot or so in thickness, occur locally. Often they are laminated and their lower and upper surfaces broadly undulatory. A very large proportion of both the small rock fragments and the large blocks is porous. "Breadcrust bombs" with dark glassy crusts, and broken fragments of these, are locally abundant. Pale pumice is also to be found locally.

As a result of my examination of the debris of the *nuées ardentes** of the 1929-32 eruptions of Mt. Pelé, which occupies the Rivière Blanche or "Avalanche" Valley, I found that the above description applies in every detail to the Martinique deposits, where the porosity of the rock-fragments is presumably to be correlated with the prolonged gas-emission that Perret and others regard as essential for eruptions of the *nuée ardente* type. There would therefore seem to be no doubt at all that the latest eruptions of the Soufrière Hills volcano of Montserrat were very largely of the *nuée ardente* type characteristic of Mt. Pelé.

Petrographically the grey to pale grey andesitic rocks of Montserrat are much more conspicuously porphyritic than those of Pelé. The phenocrysts include feldspar, hornblende, and pyroxene.

The fact that the Soufrière Hills volcano is younger than the Centre Hills is at once suggested by its less mature system of drainage (see Admiralty Chart). The field relations of their rocks confirm this supposition. On the north, smooth slopes of ash and agglomerate sweep down from the nucleus of the Soufrière Hills, and their loose volcanic deposits stop abruptly against a ridge that extends from near Hodge's in the west, to Hill 965 near Tuit's, fig. 7, Plate 9. East of this they sweep down northwards towards Trant's, fig. 3, Plate 7. The ridge, and the hilly ground to the west of Bethel and Trant's, are composed of andesitic rocks, mainly in the form of well-consolidated agglomerate, that differ in character from those of the Soufrière Hills.

Considerable attention was devoted to the densely forested ground lying immediately to the east of the highest part of the summit area of the Soufrière Hills (between Hills 3002 and 2725), where, according to Mr. T. Savage English, there is a large crater breached on the east and containing a central plug. The latter forms a conspicuous forest-clad hill with one

* Incandescent avalanches of gases, rock-fragments, and dust.

or two rocky pinnacles, and rises to about 2530 ft.; it is known as Castles Peak, or sometimes as Castor and Pollux (Plate 7).

Castles Peak, so far as can be seen, is all composed of conspicuously porphyritic andesite of a pale grey colour. A continuous deep wooded valley separates it on the north, the west, and the south from some of the main ridges of the Soufrière Hills, which are regarded by Mr. English as crater walls, and extend from Hill 1885 through Hills 2725, 3002 (Chance's Mountain), 2860, and 2600. The crater has thus a diameter of a little less than three-quarters of a mile, and is supposed to be breached between Hills 2660 and 1885. The rock forming the encircling ridges can be seen only where earthquake rock-falls have removed the vegetation. The ridges are partly formed of agglomerate, and partly of andesite less conspicuously porphyritic than that of Castles Peak. This andesite is presumably intrusive, for no corresponding lava flows have been found and the precipitous slopes of the rock walls show no signs of stratification.

During my visit to Martinique I examined the summit of Mt. Pelé with its great composite "dome" of andesite in the old crater of 1902. There are very considerable resemblances between English's crater, with its central andesite hill, and the present configuration of the summit area of Mt. Pelé. Although the analogy is not complete, I regard Mr. English's interpretation of the forest-clad Montserrat topography as almost certainly correct. It seems impossible to account for the deep valley that encircles Castles Peak on the north, west, and south as merely a product of erosion, Plate 7, and fig. 5, Plate 8. Like the shallower "fosse" of Mt. Pelé, fig. 4, Plate 8, it appears to have been formed by an andesite "dome" rising within a crater. A deep fosse-like valley was thus produced between the slopes of the rising dome and precipitous encircling crater walls. Details of the differences in the present conditions of the Pelé and Soufrière Hills craters will be reserved for a more detailed report.

Chance's Pond, which lies in a comparatively slight depression in the summit ridge of the Soufrière Hills about 200 yards north-east of the top of Chance's Mountain, was a small reedy swamp when I visited it after a prolonged drought. No rock is seen near it, owing to the dense forest growth, and I agree with Sapper that there is no indication of the pond being a crater lake.

St. George's Hill—As indicated by previous observers, St. George's Hill (1200 ft.) proves to be a small separate volcano. The steeply dipping deposits of its flanks include much pale pumice. Various lines of evidence suggest that the volcano is older than the latest eruptions of the Soufrière

Hills, but this conclusion cannot be regarded as certain. It is clearly younger than the Centre Hills, for on the north its deposits overlie agglomerate belonging to that volcano.

Centre Hills—The Centre Hills (2450 ft.; fig. 3, Plate 7, and fig. 9, Plate 9) and the surrounding lower ground represent a large maturely dissected volcano, the deposits of which extend southwards to Old Road Bay on the west coast and Trant's Bay on the east, and northwards to about the latitude of Cars Bay. On both leeward and windward coasts, its well-bedded agglomerates and tuffs form precipitous iron-stained cliffs.

Inland exposures of the lower slopes of the volcano, along road and track, are for the most part in tumultuous agglomerate containing more or less matrix of volcanic dust or sand, and give little indication of the essentially well-bedded nature of the deposits as exposed in the coastal cliffs. Here it is seen that irregularly lenticular beds of fine volcanic tuff or sand are intercalated in the structureless agglomerates. Layers of pale pumice are especially prominent in both coastal and inland exposures of the northern part of the volcano. On the whole, the deposits are more consolidated than those of the Soufrière Hills.

The coarse agglomerates contain andesite fragments of all sizes. Large blocks occur in them locally and, as in the Soufrière Hills, many of them are scattered over the cultivated slopes. The andesites usually contain small fine-grained igneous xenoliths. Many of the rock fragments and boulders are greyish-green to reddish porphyritic andesites with a fine-grained ground mass, and are readily distinguishable from the coarser-grained grey andesites of the Soufrière Hills. In the field the porosity so characteristic of the large boulders and small rock fragments of the Soufrière Hills was found to be only locally conspicuous in the volcanic products of the Centre Hills.

The densely forested mountain nucleus of the Centre Hills volcano was not very thoroughly examined. Enough was seen, however, to make it clear that no old crater is preserved in recognizable form, and that, in addition to agglomerate, a good deal of solid andesite (presumably intrusive) enters into its composition. Massive olivine-bearing andesite was found at the eastern margin of the Centre Hills area north of Tuit's and south-west of Trant's, where its mode of occurrence (extrusive or intrusive) is rather doubtful.

The phenocrysts of the andesitic rocks of the Centre Hills always include feldspar and pyroxene; hornblende occurs in addition in many places, and olivine is found at certain localities.

Garibaldi Hill (840 ft.) on the coast north-west of St. George's Hill, is a small volcanic centre belonging most probably to about the same eruptive period as the Centre Hills volcano.

Silver Hill, forming the northern end of the island, is a small maturely dissected volcanic mass rising to 1285 ft. The drainage system is radial and the slopes deforested, fig. 9, Plate 9. Its rocks are well exposed inland and finely displayed in coastal cliffs. They consist to a considerable extent of solid andesite, which passes locally into agglomerate or explosion breccia, usually without any sign of stratification. Most of the rocks forming Silver Hill thus contrast strongly with the well-bedded products of the other volcanic centres. They probably represent a relatively deep-seated volcanic nucleus from which the accompanying bedded tuffs and agglomerates have been almost everywhere removed by long-continued erosion. In hand specimens the andesites of Silver Hill closely resemble some of those of the Centre Hills. They are pyroxene-andesites that locally show traces of hornblende.

At one locality, just west-north-west of Pinnacle Rock, a hollow has been filled by a series of flat-bedded tuffs and pumiceous volcanic sands or ashes of types similar to those of the most northerly exposures of the Centre Hills deposits, as seen, for instance, in cliffs on the west and east coasts just south of the latitude of Cars Bay. The unconformable relation of these flat beds to the rocks of Silver Hill is clearly seen at the top of the cliffs near Pinnacle Rock. The presence of this isolated patch, or outlier, of fragmental materials derived from the Centre Hills volcano clearly establishes the earlier date of the Silver Hill eruptions.

A feature of the Silver Hill area is the presence of a number of centres of former fumarolic activity. One of these dead *soufrières* was noticed by Dr. Jaggar as long ago as 1902 while he was passing the island in a steamer. The sites of these half-dozen old *soufrières* are conspicuous from the sea because of the pale colour assumed by the country rocks where they have been altered by the volcanic gases. Springs said to be "salt" to the taste are known near the shore close to two of the old *soufrières*.

THE ACTIVE SOUFRIÈRES

A summary of data* regarding the active *soufrières* of the island is given in Table I. Only Galway's and the two Gage's *Soufrières* have been mentioned in published descriptions of Montserrat, and the map, fig. 1, is the first on which the position of any *soufrière* has been shown.

* The geographical distribution of the active *soufrières* has been considered above in the "Summary of Volcanic History" (p. 239).

The more important *soufrières* (Gage's Lower, Galway's, and Gage's Upper) are situated between about 900 and 1600 ft. above sea level.

It is not proposed to give in this preliminary report any details of the gases emitted* or of the minerals formed at the *soufrières*. A very general account is sufficient. The most obvious product at all the centres of activity is hydrogen sulphide. The unpleasant smell of this gas is only too evident to the leeward of the orifices, and during times of maximum seismic activity in recent years it has caused considerable discomfort in Plymouth. Even during the comparatively quiet period of my stay in the island, temporary concentrations of the gas in the capital were strong enough to be decidedly unpleasant. In my opinion, Gage's Lower *Soufrière* and Galway's *Soufrière* were the main sources of the gas that reached Plymouth. The emission of hydrogen sulphide is strictly confined to the sites of the *soufrières* listed in Table I.

At all localities the gases come up through agglomerate of the *Soufrière Hills* volcano. Owing to the fact that the fumes kill the local vegetation and that mineral changes brought about in the country rock render it locally pale to pure white in colour, the sites of the *soufrières* are very conspicuous. All steam and gas jets are encrusted with yellow sulphur, but the total amount of this mineral to be seen at the present day is not great. At one time sulphur is said to have been exported in barrels from Galway's *Soufrière*.

The decomposed "breadcrust bombs" reported by Mr. Perret from Gage's Lower *Soufrière* have in my opinion been derived from the adjacent altered agglomerate, and do not indicate any local explosive activity. Breadcrust bombs are abundant elsewhere in the agglomerates of the *Soufrière Hills* volcano (p. 243).

During the recent troubles Gage's Lower *Soufrière* (fig. 8, Plate 9) has been under constant observation by Mr. Perret and local officials of the Montserrat Department of Agriculture. At the present time gas and steam are emitted under greater pressure at Galway's than at Gage's, but only at the latter *soufrière* do the gases noticeably affect the eyes and throat. When Mr. Perret visited Gage's Lower *Soufrière* in May 1936, he was at once struck by the marked decrease of activity as compared with that observed during his previous visits.

The temperatures recorded at the *soufrières* continue to be comparatively low from a volcanological point of view. There appears to have been little or no rise of temperature either at Gage's Lower *Soufrière* or at Galway's. The thermograph at Gage's, it is true, has been record-

* Some information regarding the gases has been published in Mr. Perret's reports to the Governor of the Leeward Islands.

TABLE I—ACTIVE SOUFRIÈRES OF MONTERRAT

Name of Soufrière	Date of origin and record of activity	Location referred to summit of Chance's mountain	Description (1936)	Temperature of steam jets (1936)	Temperature of bubbling water (1936)	Remarks
Galway's	Known in 1810. Activity probably increased during both 1897-1902 and 1933-6 earthquake periods	$\frac{1}{4}$ mile S.S.E.	In large amphitheatre-like depression near head of White River Ghaut. Large steam and gas blowhole, small steam and gas jets, and hot springs	98° C. to 115° C.	72° C. to 92° C.	Also called Galloway's, South, or Roche's Soufrière. Probably oldest active soufrière. Large area (about 5 acres) affected by gases
Gage's Upper	Very old. Activity increased just before or during earthquake periods of 1897-1902 and 1933-6	300 yards W. 21° N.	In west-north-westerly ghaut S. of Gage's, and in shallow side-ravine in its south bank. Small steam and gas jets and hot springs	93° C. to 95° C.	—	Quite extensive area in south bank affected by gases. Temperature of hot springs some way down main ghaut 53° C. to 58° C.
Cow Hill	Very old. Activity probably renewed, or increased slightly, just before or during 1933-6 earthquake period	1 mile E. 42° N.	In steep slope of northern bank of first ghaut S. of Tar River estate buildings. Small steam and gas jets	—	None	Also called Tar River Soufrière. Some years ago described by Mr. English as "long extinct"

Gage's Lower After 1896 cloudburst. Unusually active during 1897-1902 and 1933-6 earthquake periods			
	Almost $\frac{1}{2}$ mile W. 15° N.	In short deep side-ravine in north bank of west-north-westerly ghaut S. of Gage's. Numerous small steam and gas jets and (sometimes) hot springs	112° C. to 115° C. by thermograph. 95° C. by maximum thermometer
			82° C. Dried up by middle of May after long drought
			Often called simply "Gage's Soufrière". Large area affected by gases. Thermograph readings possibly too high. Gases affect eyes and throat
Mulcair	? between 1896 and 1899. More active since about 1933	At base of cliff on each coast, just S. of Hell Hole Bay. Emission of hydrogen sulphide gas	— Accessible only by boat on a calm day. Not seen at close quarters by expedition
Cow Hill New	? about 1933 or 1934.....	In bottom of dry ghaut a few hundred years S.W. of Cow Hill Soufrière. Emission of hydrogen sulphide gas	None Located 1936. Area affected by gases is small (1250 sq. yds.)
Spring Ghaut	? 1933 or later	In bottom of Spring Ghaut near its head. Emission of hydrogen sulphide gas	None Located 1936. Area affected by gases is very small

ing temperatures up to about 10° higher than in 1935, but the instrument has proved unreliable in the past and this may quite possibly account for much of the difference between its readings and those obtained with an ordinary maximum thermometer held in a steam jet (e.g., 95° C.). At Galway's the maximum temperature recorded at the main blow-hole depends on how far the thermometer can be pushed down the orifice against the gas pressure. A temperature of 120° C. was recorded at Galway's in 1935.

It is clear from past records that the general activity of the Montserrat *soufrières* underwent a marked increase at the time of the 1897–1902 and the 1933–6 series of earthquakes. According to local observers, each seismic crisis of the recent earthquake series was accompanied by a noticeable temporary increase in the emission of hydrogen sulphide gas at Gage's Lower *Soufrière*.

After the eruptions of Mt. Pelé and of the *Soufrière* of St. Vincent in 1902 earthquakes ceased in Montserrat and the activity of the *soufrières* died down. The present period of earthquakes and *soufrière* activity, on the other hand, did not *begin* until *after* the Mt. Pelé eruptions of 1929–32.

HOT SPRINGS

At two localities in the island hot springs occur unassociated with any *soufrière* activity.

Two are found close together in the Tar River—a permanent stream fed by a cold spring—about 500 yards S. 30° E. from Tar River estate buildings. Each spring is associated with stalactitic deposits. The maximum temperature found was 40° C.

The other hot springs feed Hot Water Pond, a shallow pool held up by beach sand at the mouth of a small ghaut on the west coast, one mile north-west of Plymouth. The water is locally just too hot to hold the hand in with comfort.

CONCLUSION

The salient facts in the geological history of Montserrat have been set out above. There is no possible doubt that its localized and recurrent *soufrière* and earthquake activity are related phenomena of volcanic origin connected in some way with the presence of molten or semi-molten magma (lava) below the *Soufrière Hills*, the most recently active volcano of the island. The eruptions of this volcano were of the *nuée ardente* type characteristic of Mt. Pelé and other volcanoes of the Lesser

Antilles. It is almost certain that Castles Peak represents a Peléan dome in a breached and eroded crater. The active soufrières are not closely associated with this old crater.

The eruptions of the Soufrière Hills volcano took place in Pleistocene or Recent times, that is to say within the last 1 or 2 million years. Since the last eruptive period erosion has been very considerable. It would thus appear that after the date of these volcanic outbursts a long period—to be measured possibly in thousands of years—elapsed before the island first became known to the civilized world (1493). Since that date no eruptions have been recorded. My observation of other islands was limited and incomplete (p. 232), but of the inactive West Indian volcanoes I have seen I should say only the well-preserved cone of Nevis looks definitely younger than the Soufrière Hills. Descriptions and pictures of the cones of Saba and of the Quill in Statia lead me to believe that they also are less eroded.

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DESCRIPTION OF PLATES

PLATE 7

- FIG. 2—Castles Peak and English's Crater from the north-east. Castles Peak, the central forest-clad hill with two rocky pinnacles, is regarded as a Peléan dome within the breached crater. The "fosse" between the dome and the crater walls completely encircles the far side of Castles Peak.
- FIG. 3—View across part of English's Crater, looking north-eastwards from ridge near summit of Soufrière Hills. The photograph shows clearly the "fosse" between the precipitous crater wall on the left and the slopes of Castles Peak on the right. In the background, on the left, rise the cloud-capped Centre Hills and, on the right, the *nuée ardente* deposits of the Soufrière Hills slope gently down to the sea near Trant's.

PLATE 8

- FIG. 4—View looking north-north-westwards along the "fosse" of Mt. Pelé towards Morne La Croix. Slope of new dome (1929–32) on left; crater wall on right.
- FIG. 5—View looking southwards in "fosse" of English's Crater. Slope of Castles Peak dome on left; crater wall on right.
- FIG. 6—Agglomerate cliffs on east coast of Montserrat. View looking southwards from the mouth of Tar River. In the foreground is typical Soufrière Hills agglomerate formed by eruptions of *nuée ardente* type. In the distant promontory are seen the steeply inclined fossiliferous tuffs of Roche Bluff.

PLATE 9

- FIG. 7—Soufrière Hills from the north. View taken from a ridge (right foreground) forming part of the Centre Hills volcano. Note how a smooth slope of ash and agglomerate sweeps down from the mountainous nucleus of the Soufrière Hills and stops against the older rocks of the Centre Hills ridge. Outline of the summit of Castles Peak stands out against the clouds towards the left of the skyline.
- FIG. 8—View of Soufrière Hills, looking eastwards from the road south of St. George's Hill. Earthquake landslips show up as pale streaks on the steep slopes above the figure in the foreground, and the cliff behind Gage's Lower Soufrière stands out as a pale patch lower down and to the left.
- FIG. 9—View of Centre Hills, looking south from lower slopes of Silver Hill. The track leads southwards to Gerald's, where it joins a motoring road.
-



FIG. 2.



FIG. 3.



FIG. 4.

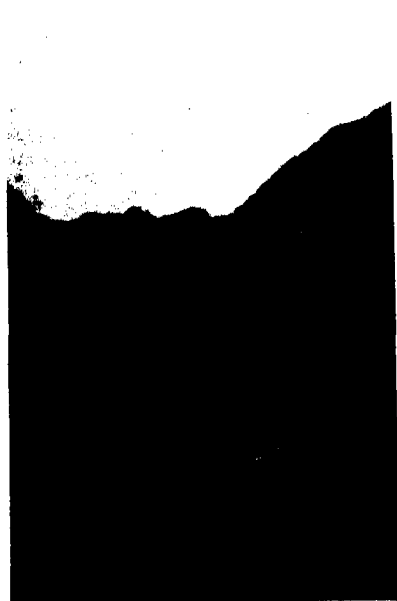


FIG. 5.



FIG. 6.



FIG. 7.

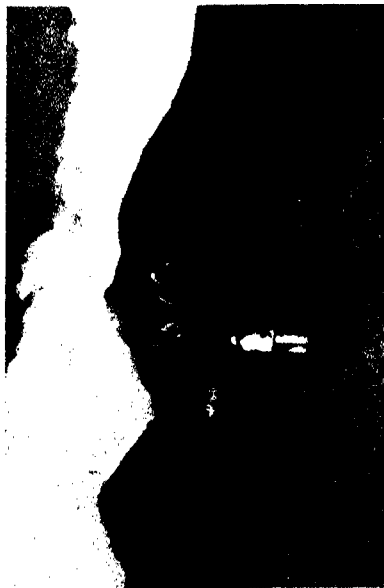


FIG. 8.



FIG. 9.

The Relation of Pathogenic Viruses to the Cells of Their Hosts

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(Received 19 August, 1936)

It is now generally assumed by the majority of workers in the field of virus diseases that the term "virus" should be limited to those small organisms, either approaching or below the limit of ordinary microscopic visibility, which are incapable of multiplication *in vitro* except in the presence of living animal or vegetable cells.

This limitation is obviously only provisional, as with a fuller knowledge of the conditions obtaining in the living cell it may at some time become possible to simulate these sufficiently closely to allow of the growth of the virus in a cell-free medium. Until this is accomplished, however, the presence of the living cell must be regarded as essential, and we are faced with the problem of elucidating its role in the multiplication of the virus.

On the basis of the facts at present available, the simplest and most satisfying hypothesis appears to be that which regards the virus as an obligatory parasite, multiplying either in or on the living cell. The intracellular changes so frequently observed in the lesions of animals and plants infected with various viruses seem to point very definitely to the cell as the site of multiplication, but do not indicate whether this is to be regarded as taking place actually within the cell or on its surface. It is true that in the Rickettsiae the process of multiplication in the interior of the cell can be followed by direct microscopical examination but, in view of their comparatively large size and special characteristics, these organisms fall into a special group and their behaviour in relation to the animal cell may not necessarily be the same as that of the typical "filter-passing" viruses. Unfortunately, the small size of most viruses makes it at present difficult, if not impossible, to determine their location by microscopical observation, and it is only with one or two of the larger of these that the examination of stained preparations has yielded promising results.

A possible line of attack was suggested by certain observations made in the course of an investigation of the photodynamic action of methylene blue on the bacteriophage (Perdrau and Todd, 1933, *a*) and subsequently on a number of pathogenic viruses (Perdrau and Todd, 1933, *b*).

With a staphylococcus phage it was found that, while the free phage was highly sensitive to the photodynamic action of methylene blue, it became very much more resistant after union with the corresponding bacterium. This protection of the phage was only exercised by *living* bacteria, the killed organisms being without effect. Similarly, certain viruses (*e.g.*, the viruses of herpes, Borna disease, and fowl plague), as they exist in freshly prepared suspensions of *living* cells from infected animals, were found to be very much more resistant to the photodynamic action of the dye than they were in cell-free filtrates, or in glycerinated infected materials where the glycerine kills the cells of the host but is without effect on the virus.

In attempting to explain the nature of this protection, it appears reasonable, in the light of the results obtained, to attribute it to an actual penetration of the phage or virus into the living cell, in the interior of which the oxidation-reduction potential is such that photodynamic inactivation, requiring as it does the presence of unreduced methylene blue, can not take place.

If this view is correct and the presence of protection really indicates the location of the virus (*i.e.*, whether it is inside or outside the cell), we have a test which should enable us to study the behaviour of the virus in the infected animal by determining the time of its entry into the various susceptible cells of the body. Naturally the explanation suggested above can for the present be regarded only as a working hypothesis, but as such it appears to invite examination, and the present communication, which is concerned only with viruses pathogenic for animals, deals with experiments directed to this end.

The present work was suggested by results obtained in some previously described experiments (Perdrau and Todd, 1933, *b*) when it was shown that, if a rabbit be inoculated with the virus of either vaccinia or herpes by scarification of the cornea, the development of the specific lesions can be prevented by the local application of methylene blue and exposure to light, but for this treatment to be effective it is essential that it should be carried out within a definite period (some 12 to 24 hours) from the time of inoculation, as after the expiry of this period treatment is entirely without influence on the progress of the infection, the existence of a latent period in the appearance of the phenomenon of protection being thus demonstrated.

Experiments of this type, where the inactivation of the virus is carried out in the living animal, while useful in showing the presence of a latent period in the appearance of protection, were not promising for further work as one could only use viruses causing lesions which were superficial

and therefore accessible to the action of light. Moreover, no accurate control of the amount of dye actually coming in contact with the virus was possible. In the experiments described below, inactivation was therefore carried out *in vitro* where these difficulties did not arise.

EXPERIMENTAL

In earlier work on a staphylococcus phage, it was found that the degree of protection afforded by contact of the phage with the corresponding staphylococcus was not absolute, and that the phage could be inactivated, even in the presence of the staphylococcus, if the irradiation was sufficiently prolonged. Investigation showed that the protecting bacterium, though very much less sensitive than the phage, was itself ultimately killed by the photodynamic action of the dye, and when this happened the protection, which is only exercised by the *living* bacteria, naturally ceased. This is a point to be borne in mind as, if the irradiation is too intense or is unduly prolonged, evidence of protection may possibly be missed through the death of the protecting cell.

In our first experiments with the brain cells of mice infected with louping ill (Perdrau and Todd, 1933, *b*) we saw no evidence of protection of the virus. Further investigation of this apparent exception to the general rule, however, has shown that failure to obtain protection was merely due to excessive irradiation. The virus of louping ill is highly susceptible to the photodynamic action of methylene blue, so that for the demonstration of protection too intense an illumination must be avoided. In working with sensitive viruses it is to be remembered that bright daylight is highly actinic in comparison with the light of a 100 c.p. electric lamp, so that the handling of irradiated mixtures and the injection of animals must be carried out in a subdued light. When the experiments were repeated under these conditions the virus of louping ill was found to behave like the other viruses which we have examined, and to show quite a definite protection. This observation incidentally points to the fact that the protection afforded by living cells to the different viruses varies much in degree. For instance, the protection afforded to the viruses of herpes and of fowl plague is greater than to that of vaccinia.

In the work described in the present communication, in view of the absence of any data on the susceptibility of the various animal cells to the photodynamic action of methylene blue, it was thought advisable to adhere to the method previously employed which had already proved satisfactory for several viruses, viz., the addition of sufficient methylene blue to a suspension of the cells in Tyrode to yield a concentration of the

dye of 1 in 10,000, and the irradiation of the mixture for half an hour at a distance of 20 cm. from a 100 c.p. pointolite lamp.

Most animal viruses can be inactivated quite easily, as they exist in filtrates or ground suspensions of preserved material where the tissue cells of the host are dead, but our observations were confined to four viruses only, namely those of fowl plague, herpes, vaccinia, and louping ill.

In order to avoid confusion with the simple phagocytosis of fragments of tissue containing the virus, filtrates were used as often as possible. An injection of a potent preparation of the virus was made into a particular organ or tissue (brain, testis, blood) and the animals were killed after certain intervals. Suspensions were made by shaking some of the infected tissues with glass beads in Tyrode's solution to which sodium citrate (0.4%) was usually added. The suspension, which contained roughly 2% of the tissue, was strained through a double layer of muslin, concentrated methylene blue added to yield a concentration of 1/10,000, and 2 cc. of the mixture, in a layer 2 mm. deep, irradiated at a distance of 20 cm. from a 100-c.p. pointolite lamp for half an hour. Under these conditions an exposure of not more than 10 minutes was sufficient to inactivate the unprotected virus in the case of each of the four viruses used. In all the experiments recorded here a control inoculation was always made to prove the infectivity of the original suspensions, and since the result was always positive these control inoculations are not recorded in the appended tables.

Fowl Plague—It will be seen from Table I that in the living animal the virus of fowl plague does not become "protected" by the blood cells of a fowl, after an intravenous inoculation, until 3 hours have elapsed since the moment of inoculation, whilst in the brain of a fowl injected intracerebrally protection by the cells of the brain is established in one hour. In the brain of the mouse the time is two hours. In the light of what has been stated, we regard these times as approximately those required by this virus to penetrate the susceptible cells of the host.

These experiments were repeated *in vitro* on suspensions of living cells of the same tissues obtained from a normal animal. A filtrate of virus was added to the suspensions and the mixture was incubated for various periods at 37° C. before irradiation. In 23 experiments with normal fowl blood apparent protection was obtained only 3 times, viz., after periods of contact of 1 hour (twice) and of 3½ hours (once). With fowl brain suspensions protection was obtained twice out of 4 attempts, both after 3 hours' contact. With the blood of other animals (man, rabbit, horse, rat, mouse, and guinea-pig), the only positive results were with rabbit's

blood which protected the virus twice in 7 attempts (in both cases after an hour's contact). These results are somewhat irregular, but suggest that it is not easy to infect *in vitro* resting adult cells, even those of a susceptible animal. The two positive results in the rabbit—a non-susceptible animal—might be an indication that an inapparent infection is possible in this animal. When instead of resting tissues those of an actively growing chick embryo were used, the result was quite clear-cut, as seen in Table I. Here protection was established in half an hour.

The tests consisted of intramuscular inoculations into young or half-grown chicks.

TABLE I—FOWL PLAGUE. LATENT PERIOD IN APPEARANCE OF PROTECTION IN THE LIVING ANIMAL

Tissue	Time of contact	Protection
Blood of fowl (intravenous)	1 hour	0
“	1 “	0
“	2 “	0
“	3 “	+
“	4 “	+
“	4 “	+
Brain of fowl	$\frac{1}{2}$ “	0
“	$\frac{1}{2}$ “	0
“	1 “	+
“	5 “	+
Brain of mouse	1 “	0
“	2 “	0
“	2 “	+
“	3 “	+
“	4 “	+
Latent period <i>in vitro</i>		
Chick embryo	$\frac{1}{2}$ hour	0
“	$\frac{1}{2}$ “	+
“	1 “	+
“	3 “	+
“	3 “	+

Vaccinia—A broth filtrate was used in these experiments, which were made on rabbits. The virus was inoculated into the testis in the majority of experiments and suspensions of the organs were treated subsequently in the manner previously described. It will be seen from Table II that the earliest time in which protection was obtained was after a contact of 10½ hours. With chick embryo *in vitro* the virus was not protected any

earlier, although a culture strain of the virus maintained on minced chick embryo was used.

With normal adult tissues *in vitro* no protection was obtained in 25 hours, the longest period tested.

The tests consisted of intradermal inoculations into the shaved skin of the rabbit, and the results were read on the fourth day.

TABLE II—VACCINE VIRUS. LATENT PERIOD IN APPEARANCE OF PROTECTION IN THE LIVING ANIMAL (RABBIT)

Tissue	Time of contact	Protection
Peritoneum	4 hours	0
Testis	4 "	0
Spleen	6 "	0
Testis	6 "	0
"	10½ "	+
"	10½ "	+
"	14 "	0
Peritoneum	14 "	+
Testis	14 "	+
"	18 "	?
"	18 "	+
"	18 "	+
"	24 "	+

Herpes—The procedure adopted with the virus of herpes was similar to that used for vaccinia except that the supernatant fluid of a centrifuged suspension of glycerinated brain was used instead of a filtrate. The filtrates of herpes virus in our experience only just stand dilution to 1 in 1000 and would therefore not be capable of the dilutions which these experiments necessitate. In the testis of the living animal (Table III), the earliest time at which protection was obtained was after a contact of 18 hours. Had filtrates of herpes virus as potent as those of vaccine virus been available, it is quite possible that a shorter latent period might have been observed. The tests consisted of intracerebral inoculations into rabbits.

Louping Ill—As already stated, we have been able to obtain protection of louping ill virus in living cells (mouse brain) provided the irradiation is not too intense or prolonged.

With a culture of louping ill virus maintained on chick-embryo brain in Tyrode's fluid we have found that protection is established as early as one hour after contact at 37° C. (Table IV). Our tests were made by intracerebral inoculation in mice.

TABLE III—HERPES. LATENT PERIOD IN THE APPEARANCE OF PROTECTION IN THE LIVING ANIMAL (RABBIT)

Tissue	Time of contact	Protection
Testis	10 minutes	0
"	2½ hours	0
"	14 "	0
"	18 "	0
"	18 "	+
"	18 "	+
"	24 "	+
"	24 "	+
"	24 "	0

TABLE IV—LOUPING ILL—LATENT PERIOD IN APPEARANCE OF PROTECTION IN CHICK EMBRYO BRAIN *in vitro*

Tissue	Time	Protection
Brain	½ hour	0
"	1 "	0
"	1 "	+
"	3 "	+
"	5 "	+
"	5½ "	+
"	24 "	+

DISCUSSION

The protection of organisms, situated in the interior of living cells, from the action of destructive agents in the surrounding medium is perhaps not surprising and has been demonstrated for bacteria by Rous and Jones (1916) who showed that typhoid bacilli ingested by guinea-pig's leucocytes were protected both from the action of normal rabbit's serum, which is highly bactericidal for this organism, and also from the lethal action of potassium cyanide to which the bacillus is more sensitive than the leucocyte. In the same way the red blood corpuscles of the rat were protected from the action of an anti-rat serum (prepared in the dog) when phagocyted by the dog's mononuclear leucocytes.

An interesting study of the fixation and protection of viruses by cells has quite recently been made by Rous, McMaster, and Hudack (1935), who worked with cells obtained by the tryptic digestion of rabbit embryo tissue cultures, and the viruses of vaccinia, and of Shope's infectious fibroma of rabbits. They found that both viruses rapidly became fixed upon cells freed as individuals and submitted to the virus in suspension. This happened whether the cells were alive or had been killed by heat or

ultra-violet light. When cells carrying the virus were exposed to neutralizing antisera the virus was not significantly affected if the cells were living, whereas if they were dead the activity of the virus was nullified. In discussing these results they make the following comment: "One is tempted to suppose that the viruses used in the present experiments, after becoming attached in some way to living cells, are taken into the latter and owe their persistence in active state to an intracellular situation. But the data do not justify this supposition. They prove only that the protection of the viruses is in some way dependent upon cell life. The maintenance of a special state of affairs at or near the cell surface might suffice for protection".

This appears to be a legitimate conclusion from the results obtained by these observers but, in view of the fact that we find that the union of vaccinia virus with the cell in the living animal, as shown by the appearance of protection from the lethal photodynamic action of methylene blue, requires a minimum contact of 10½ hours, and as Rous, McMaster, and Hudack only allowed a contact of from one-quarter to 1 hour, we are inclined to believe that they were dealing rather with the action of immune serum on an adsorbed virus and that their results, while of the greatest interest from that point of view, do not concern our immediate problem here. This criticism also applies to the work of Rivers, Haagen, and Muckenfuss (1929) on the protection of the virus of vaccinia from the action of antivaccinial serum.

Haagen (1933) found that the virus of yellow fever is quickly neutralized *in vitro* by immune serum in the presence of living cells of fowl embryo but that, if the virus is allowed to remain in contact with the cell for 3 hours at room temperature before the addition of the immune serum, the virus survives and multiplies in the tissue cultures. We have not studied the virus of yellow fever and are therefore not in a position to offer any opinion as to the nature of the protection observed in this case, but would draw attention to the difference between the photodynamic inactivation of a virus, which is a true inactivation, in the sense that it is an irreversible process, and "inactivation" by means of an antiserum, which under certain circumstances (*e.g.*, if the time of contact is not unduly prolonged) is reversible, and may be regarded rather as an inhibition of the activity of the virus than an actual inactivation.

In an earlier communication (1933, *b*) we considered the possibility that a phage or virus might be protected by conditions existing in the immediate neighbourhood of the living cell, but saw no adequate evidence of any such action.

The lethal photodynamic action of methylene blue is certainly a process

of oxidation and the reduced dye is quite inactive, so that if the rH in the interior of a living cell is sufficiently low to maintain any methylene blue entering it in a reduced condition, a phage or virus located actually in the cytoplasm of the cell must be protected so long as these reducing conditions are maintained, *i.e.*, during the life of the cell.

Although we have no exact knowledge of the oxidation-reduction potential existing in the interior of mammalian cells, a certain amount of information concerning other cells is available. Carefully controlled work on the oxidation-reduction potential of the living cell was carried out by Cohen, Chambers, and Reznikoff (1927-28) on *Amoeba dubia* and *Amoeba proteus* by the micro-injection of dyes. They found that the amoeba develops a high primary reducing potential, certainly not less than rH 7.6, and that in the presence of the neutralizing effect of atmospheric oxygen the internal rH appears to assume any value between 13 and 18, depending on secondary factors. If a small injection of a 1% aqueous solution of methylene blue is made into the cytoplasm of the cell the dye is decolourized in about a minute and the injection of ferrocyanide brings back its colour.

A very striking fact in connexion with the phenomenon of protection is the latent period which precedes its appearance. This differs widely in different viruses, but, at any rate for fowl plague, vaccinia, louping ill, and herpes to which our attention has been specially directed, appears to be approximately constant for each particular virus, and it is interesting to consider its probable significance.

If, for example, a vaccinia filtrate (which in the absence of living cells is very rapidly inactivated by the photodynamic action of methylene blue) is injected into an animal, the virus remains sensitive to the action of the dye for many hours, though all the time in intimate contact with the living cells of the animal body, and it is not until it has been in contact with the cells for at least 10½ hours that it becomes protected from the lethal action of the dye. If this protection were due merely to the influence of the cell on its immediate surroundings one would have expected protection in a much shorter time, and the comparatively long latent period suggests that the virus has actually to penetrate the cell if it is to be protected.

Interesting evidence bearing on this point is provided in a recent communication of Herzberg (1936) who, by means of preparations stained with Victoriablau, has studied the development of vaccinia virus in the infected chorio-allantoic membrane of the fowl's egg. He found that elementary bodies could first be recognized in the cell in small numbers 12 hours after infection, and that after 16 to 18 hours a large number were present in many of the cells.

The agreement of these times with the latent period in the appearance of protection found in our experiments is sufficiently close to suggest that it is not merely a coincidence.

This observation of Herzberg, taken in conjunction with those recorded here, confirms the general view that multiplication of the virus does not take place until it has actually penetrated the living cell, and that mere contact of the virus with the cell is not sufficient to allow of multiplication.

We have made no attempt to determine the length of the latent period with any special precision, and it is indeed doubtful if the conditions imposed by the nature of the experiments would allow of any great degree of exactitude. In intracerebral inoculations, for example, the amount of infective material which can be injected is necessarily small, and an indeterminate amount of the virus is presumably removed from the site of inoculation by the body fluids, so that in a suspension of the brain made shortly after the injection and before any multiplication of the virus has taken place, the concentration of virus cannot be very great, particularly for viruses which do not yield highly infective filtrates. It is, moreover, not constant. The latent period of any particular virus is undoubtedly influenced to a certain extent by the tissue or organ into which the injection is made, but it is quite clear that it is the virus itself, and not the tissue, which is the main factor in determining the length of the period. It is to be noted, however, that in the rapidly growing cells of the chick embryo, the latent period seems to be somewhat shorter than in the cells of the living adult animal, at any rate for fowl plague and louping ill. A comparison of the sizes of the viruses and of their latent periods with the incubation periods of the diseases to which they give rise is interesting (Table V).

TABLE V—COMPARISON OF SIZE OF VIRUS, SHORTEST LATENT PERIOD OBSERVED, AND INCUBATION PERIOD

	Size in $m\mu$	Latent period	Incubation period
Fowl plague	75	$\frac{1}{2}$ hour (chick embryo)	$1\frac{1}{2}$ –2 days
Louping ill	20	1 " "	4–5 days
Vaccinia	150	$10\frac{1}{2}$ " (rabbit)	3–4 "
Herpes	120	18 " "	3–4 "

There does not appear to be any correlation between these various characters except that the two small viruses have much shorter latent periods than the two larger ones.

The difficulty which has been experienced in infecting the resting cells of susceptible adult animals *in vitro* is somewhat remarkable, especially

in view of the ease with which the growing cells of an embryo can be infected, and suggests the possibility that infection may only take place in cells which are actually either in process of division or are in a condition of active growth.

If this is so the fact that multiplication of a bacteriophage takes place only in the presence of actively multiplying bacteria offers an interesting analogy.

The thanks of one of the authors (C. T.) are due to the Medical Research Council for the facilities afforded at the National Institute for Medical Research, Hampstead.

CONCLUSIONS

1—The majority of viruses, as they exist in filtrates or in other fluids free from living animal cells, are easily inactivated by the photodynamic action of methylene blue.

2—The same viruses, as they exist in freshly prepared suspensions of living cells from infected animals, are very much more resistant to the photodynamic action of the dye than they are in cell-free filtrates.

3—This "protection" of the virus, which is only exercised by the living cell, appears to be due to the actual location of the virus within the cells of the host, where the virus is protected from the photodynamic action of the dye as long as the cells remain alive.

4—A virus injected into a susceptible animal remains for a time sensitive to the photodynamic action of methylene blue, and it is only after the expiration of a certain latent period that it becomes protected from such action. The latent period can thus be regarded as a measure of the time required by the virus to penetrate the cell.

5—The latent period in the onset of protection varies within somewhat wide limits for different viruses, but seems to be approximately constant for each virus, and not to be greatly affected by the tissue or organ infected. For the four viruses examined the approximate times were as follows:—

Fowl plague (fowl, mouse, chick embryo)	$\frac{1}{2}$ –3 hours.
Louping ill (chick embryo)	1 hour.
Vaccinia (rabbit)	10–14 hours.
Herpes (rabbit)	18 hours.

6—The difficulty experienced in obtaining protection *in vitro* with the living resting cells of adult animals and the comparative ease with which this is accomplished in the growing tissues of the chick embryo suggest that

infection of the cell takes place preferably in cells which are either actually dividing or are in a condition of active growth.

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The External Mechanics of the Chromosomes

I—THE SCOPE OF ENQUIRY

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(Communicated by Sir Daniel Hall, F.R.S.—Received 15 April, 1936)

[PLATE 10]

1—PREMISES

The movement of chromosomes may be regarded in two kinds of relationships according as it involves changes of shape and changes of position. The first are due to movements within the chromosomes, and may be used to infer their *internal mechanics*. The second are due to movements between chromosomes, and may be used to infer their *external mechanics*.

Many experiments have been devoted to elucidating the principles of the external mechanics, and they have been successful in showing certain essential properties of the cell outside the nucleus, particularly of the spindle and the spindle-determining bodies or centrosomes. But, when applied to the chromosomes, artificial treatment has the drawback that in making one primary change it sets up a series of secondary changes whose importance cannot be accurately assessed; comparison is therefore

vitiated. The cytoplasm and, in the resting nucleus, a semi-permeable nuclear membrane separates and protects the chromosomes from external stimuli. Thus micro-dissection and etherization have clear-cut effects on chromosome movements, but these may be secondary consequences of an action upon the cytoplasm. The value of experimental tests being thus limited, we are thrown back on the comparison of the behaviour of chromosomes in different natural circumstances. The number of variables is then under genetical control and the method of inference depends on genetical assumptions. But the necessary assumptions are now fairly well defined. We take it that the behaviour of the chromosomes depends on three variable factors: genotype, environment, and permanent structure, and there are usually means by which we can eliminate differences due to two of these. The characteristic differences between different species and varieties, for example, are due to differences of genotype, and they fall for the most part within a narrow range, the narrower the smaller the systematic group concerned. Even the most significant change that has arisen in the evolution of the genetic mechanism, the change from mitosis to meiosis, can be represented as the consequence of a shift in the time-coordination between external and internal factors in the development of the chromosomes, a *precocity* of the one relative to the other.

The only one of the older hypotheses of external mechanics that seems to have borne fruit (after long years of neglect) is Lillie's electrostatic hypothesis (1905, *a* and *b*, *et al.*). Lillie supposed that the chromosomes were distributed evenly in the nucleus and on the metaphase plate as a result of repulsions, and these repulsions were to be expected in view of the probable surface charges on the chromosomes. Gates (1909) has also pointed out that a repulsion hypothesis would apply to his observations on *Oenothera*. Lillie's hypothesis is in strict agreement with the theory of terminalization that I have developed. It was founded, like the one to be considered, chiefly on comparison of natural observations, not on experiment. It seemed to break down over two main difficulties: the first that of relating the mechanics of meiosis (with pairing) and mitosis (without pairing); the second that of reconciling metaphase repulsion from the pole with apparent anaphase attraction to the pole.

These difficulties have now been overcome, with the help of three kinds of new observations. The first are those which suggest that specific attractions and non-specific repulsions are the forces moving chromosomes equally at meiosis and mitosis, and equally at prophase and anaphase. The precocity theory which expresses this unification of principle removes the internal inconsistencies from the system and paves the way for a

mechanical analysis. The second are the observations of Kühn and the experiments of Belar and Fankhauser. They provide the data, with regard to the mitotic spindle, which are essential for considering chromosome mechanics. The third are the observations of the continuity in form and function of the "spindle fibre attachment chromosome", or *centromere*, as it may more conveniently be described. Its action in producing the movement of terminalization in the undifferentiated substrate of the nucleus enables us to understand its more efficient action in the differentiated substrate of the spindle.

It is now, therefore, possible to state the assumptions in terms of which the external mechanics of the chromosomes, in their most widespread type of behaviour, may be analysed, thus:

1—The chromosome threads attract one another by virtue of a specific attraction between their homologous parts, the chromomeres. This attraction is largely satisfied by an association in pairs. It is optional, for it operates as a rule only during the period when the thread is extended or is assuming a spiral form, *i.e.*, in prophase and metaphase, not in anaphase and telophase. A *residual attraction* determines somatic and secondary pairing.

2—The nucleus enters prophase in mitosis after the chromosomes have divided into chromatids, so that the two attracting threads are the chromatids or daughter chromosomes in mitosis. But in meiosis prophase begins before the chromosomes have divided. It is *precocious*. The attracting threads are therefore whole homologous chromosomes from opposite parents which pair throughout their length if time permits. But in some organisms with very long chromosomes (*e.g.*, in *Fritillaria*, Darlington, 1935, *a, b, c*) pairing is never completed; it is therefore localized in the proximal part where it begins, owing to the action of a time-limit.

3—After pairing in meiosis, the chromosomes separate when they divide into chromatids; but as a result of the strain imposed by a relational coiling that develops while they are paired, the chromatids of the opposite chromosomes break at corresponding points and *cross over*. After separation, chromatids of the two chromosomes reveal an exchange of partners at the places of crossing-over, which are called *chiasmata*. The chiasmata maintain the association of bivalents by virtue of the longitudinal cohesion of the chromatids, and of the lateral attraction of chromatids, not of chromosomes (as was formerly believed).

4—Even distribution of the chromosomes in the nucleus and on the spindle is determined by a non-specific *body-repulsion* between all parts of all chromosomes. This repulsion is in equilibrium with any attraction that may be present. It is an inverse function of distance. It is there-

fore greater within closed loops (between chiasmata) than between open arms (distal to chiasmata); distal chiasmata are moved towards the ends of the chromosomes. This movement is described as *terminalization*.

5—A special repulsion is shown at meiosis between two undivided chromomeres of the partner chromosomes, the centromeres, by the expansion of the loop they lie in at the expense of its neighbours, an expansion which in extreme cases pushes the chiasmata on either side of this loop to the ends of the chromosomes, where all the chiasmata fuse. At the terminal chiasmata which arise in this way the chromosomes are held together by a special attraction of the ends, which show not merely lateral but also terminal affinity.

6—When the spiralization of the chromosomes has reached a certain stage, usually characteristic for the type of division, the nucleocytoplasmic surface which has acted as a semi-permeable membrane breaks down. A body, the *centrosome*, which like the centromeres has a different fixation-staining reaction from the chromosomes, lies outside the nucleus during these changes and divides, giving rise to two similar bodies which develop between and around them a differentiation in the cytoplasm. This differentiation consists demonstrably in a redistribution of the water in longitudinal channels and in lower concentration than elsewhere (Belar, 1929, a). This structure is the *spindle*. Its constitution is presumably determined by the orientation of chain molecules, an orientation like that of a liquid crystal, which in the nematic phase is influenced by an electro-magnetic field.

7—In the higher animals the form and behaviour of the spindle can generally be seen to depend on the number and positions of centrosomes, or spindle determinants (Boveri, 1907; Kühn, 1920; Fankhauser, 1934). But the centrosomes are invisible at all stages in some organisms, such as the flowering plants, and at some stages in most organisms. In many organisms where they are invisible, the spindle continues to behave as it does when they are seen; they must then be supposed to be present, and we can conveniently consider the spindle-determining bodies, visible or invisible, as equivalent to centrosomes, in the absence of spindle behaviour which contradicts this view. The same principles must be applied to the centromeres. They must both be considered in terms of function rather than form, since the function is evident and the form elusive.

8—The centromeres of the chromosomes in mitosis or of the bivalents in meiosis arrange themselves in an *equatorial plate* across the spindle. They are repelled by the centrosomes and by one another. The bodies of the chromosomes lie as far apart as this arrangement and their rigidity permit, exerting a body-repulsion on one another.

9—Repulsions on the spindle are more efficient in a lengthwise direction than in a crosswise direction, or in any direction in the undifferentiated cytoplasm; they are transmitted more effectively, owing presumably to the special dielectric properties of water. Paired or double centromeres are therefore bound to lie in a lengthwise direction. Hence the apparent contradiction is resolved between the paired centromeres of bivalents and the dividing centromeres of mitotic chromosomes being axially orientated and being held on the spindle, on the one hand, and the centrosomes at the poles of the spindle repelling them, on the other.

10—The shape of the spindle, which was originally related to the centrosomes alone, is now related to the centromeres also, for it is broadened in the middle as the chromosomes come to the equator and repel one another between prometaphase and metaphase, and if the chromosomes do not come to lie on the equator the spindle lengthens (Matsumoto, 1932; Dobzhansky, 1934; Smith, 1935). Its structural orientation must be established by its molecules lying in the direction in which they can transmit the repulsions most effectively. When the spindle is developed the centrosomes repel one another more strongly, and move apart unless they are prevented from doing so by the broadening of the metaphase plate. The materials available for spindle formation must be limited.

11—At the first metaphase of meiosis the centromeres of the paired chromosomes lie in an axial equilibrium with the centrosome repulsions. They move apart because the centrosome repulsions wane and the centromere-centrosome equilibrium positions therefore change. In mitosis the separation is determined by the division of the centromere, which is coordinated with the waning centrosome repulsion.

12—The centrosomes, chromosomes, and centromeres thus have three differently timed cycles of division and these cycles are differently coordinated in meiosis and mitosis. In meiosis the centrosomes are so precocious in relation to chromosomes and centromeres that they divide twice while the other bodies divide only once.

With these hypotheses in mind, it seems possible to consider the details of chromosome movements at mitosis and meiosis and use the available observations in building a coherent theory of chromosome mechanics. In order to analyse the forces at work, we must make a thorough study of a restricted group. We must know all the diverse possibilities of behaviour in that group before we draw conclusions from its diversity. We must know them in the sense that we can assign a meaning to every position and attitude and movement. For this purpose I have confined the present series of articles to *Fritillaria*, continuing my earlier observa-

tions on different aspects of its chromosome behaviour (1929-35). This genus includes within its species a relatively wide range of behaviour, and has chromosomes resembling those of *Lilium* and as much as 24 μ in length at the metaphase of mitosis.

2—MATERIAL: A SURVEY OF *Fritillaria*

Fritillaria includes species with different kinds of chromosome behaviour at meiosis. In some the chromosomes pair completely or almost completely at the pachytene stage and chiasmata are distributed throughout their length. In others the chromosomes pair only in the parts near their centromeres, and the chiasmata are necessarily also restricted to these *proximal* parts (Darlington, 1935, *a*). In a given species the number of chiasmata formed seems to be proportional to the length paired. It is therefore of interest to find out how widely this property of localization occurs amongst the different species and what other peculiarities of behaviour are associated with it.

For this purpose I have examined 25 diploid and 4 triploid forms at meiosis in the pollen mother-cells, and the results are summarized in Table I. I have divided the species into three groups, with complete localization of chiasmata, partial localization, and free distribution, respectively. The classification is arbitrary because every degree of localization is found. This indeed is to be expected, since there is great variation within the species and from chromosome to chromosome in the localization of pachytene pairing as well as in the resultant localization of chiasmata.

Applying the principle that all chromosome-behaviour can be expressed as the product of environment, genotype, and "permanent" structure, we may conclude that localization is a genotypic property, since environmental abnormality and structural hybridity can be excluded from general consideration. It will be seen that this property is distributed amongst *Fritillaria* species in all parts of the range of the genus, but is noticeably less common, and when it occurs less extreme, in the American species. It will also be seen that all the American species and one European species with free chiasmata have revealed triploid forms, while in only one of the fifteen species with localized chiasmata has a triploid been found.

It may be noted that in the species of *Fritillaria* with localized chiasmata we have a condition, analogous to that in complex-heterozygotes and other organisms that may be said to be semi-clonal, in which large parts of the chromosomes are protected from crossing-over. This condition is evidently of positive survival value since it is attained in so

TABLE I—CONSPECTUS OF *Fritillaria* SPECIES AND VARIETIES STUDIED

Species	Locality	Source	Somatic chromosomes	'ploidy	Nuclei examined	Mean chiasma frequency
<i>Localized chiasmata</i> —						
1. <i>F. ruthenica</i> Wikstr.	Caucasus	Barr, van Tubergen	18	2x	15	1.77
2. <i>F. pontica</i> Wahlenb.	Asia Minor	Merton, van Tubergen	24	2x	3	1.8
3. <i>F. meleagroides</i> Patrin	Central Asia	Elwes	24	2x	6	2.8
4. <i>F. gracilis</i> Asch. et Graeb.	Dalmatia	Elwes	24	2x	—	—
5. <i>F. meleagris</i> L.	Europe	Merton	24	2x	5	2.3
6. <i>F. latifolia</i> Willd.	Caucasus	Elwes	24	2x	—	—
var. <i>major</i>		Elwes	36	3x	—	—
7. <i>F. acinopetala</i> Boiss.	Syria	Elwes	24	2x	5	3.0
8. <i>F. hispanica</i> Boiss. et Reut.	Spain	Lawrence	24	2x	1	2.3
9. <i>F. citrina</i> Baker	Asia Minor	Barr	24	2x	4	2.2
10. <i>F. Elwestii</i> Boiss.	Lycia	Elwes	24	2x	6	3.0
<i>Partially localized chiasmata</i> —						
11. <i>F. verticillata</i> Willd.	Siberia	Boscawen	24	2x	—	—
var. <i>Thunbergii</i>		Elwes	24	2x	—	—
12. <i>F. obliqua</i> Ker-Gawl	Greece	Boscawen	24 + 2ff	2x	1	2.5
13. <i>F. oranensis</i> Pomel	Algeria	Barr	24	2x	—	—
14. <i>F. karadaghensis</i> Turill	N. Persia	Wallace	24	2x	—	—
15. <i>F. pluriflora</i> Torr.	California	Purdy	24 + 2ff	2x	1	2.4
<i>Free chiasmata</i> —						
16. <i>F. lanceolata</i> Pursh.	California	(1) Rowntree (2) Purdy	24 + 1f 36	2x 3x	—	—
17. <i>F. recurva</i> Benth.	California	(1) Barr (2) Purdy	24 + 1f 36	2x 3x	—	—
18. <i>F. pudica</i> Spreng.	California	(1) van Tubergen (2) Barr	26 39	2x 3x	—	—

19. <i>F. dasyphylla</i> Baker	Asia Minor	(1) Kew	24	2x	—
		(2) Kew	36	3x	—
20. <i>F. libanotica</i> Baker	Syria	Kew	24	2x	—
21. <i>F. pallidiflora</i> Schrenk.	S. Siberia	Kew	24	2x	—
22. <i>F. phoenanthera</i>	—	Grey (Hocker Edge)	24	2x	—
23. <i>F. askabadensis</i> Micheli	Transcaucasus ..	van Tubergen	24	2x	—
24. <i>F. Eggeri</i> Bornm.	Persia	Kew	24	2x	—
25. <i>F. imperialis</i> L.	Persia	Merton, etc.	24 + 1/12ff	2x	2.6-5.0

The names, authorities, and habitats of the species are taken from Turrill's list (1932). Many of them, however, are uncertain, since the immature flowers have had to be removed from bulbs which in some cases have not flowered again. The names are given provisionally and will be verified when possible. In the third column are the names of the growers from whom the bulbs were obtained. The sources are European except Purdy and Rowntree, who are Californian. I have to thank Lady Lawrence, Major H. J. Elwes, and the Rev. A. T. Boscawen for the gift of specimens of three species.

many different ways in different organisms, particularly by the *Stenobothrus* frequency-length relationship (Darlington and Dark, 1932). It shows that while crossing-over in some degree is generally advantageous, suppression of crossing-over or block-inheritance is also useful for part of the genes. The implications of this view I have considered elsewhere (1936, *a, b*).

The mitotic chromosome complement in *Fritillaria* varies in two structural respects. Fragment chromosomes are found in five species. They are all of the same size and appear to be supernumerary chromosomes. That is to say they are probably reduplications resulting from unequal interchange in the neighbourhood of the centromere. Such a change would also give rise to the changed complements of *F. ruthenica* ($n = 9$) and *F. pudica* ($n = 13$) which I have previously attributed to fusion and fragmentation respectively (*cf.* 1936, *b*). The typical complement of *Fritillaria* has 10 pairs with subterminal centromeres (S) and two pairs with median centromeres (M) while *F. ruthenica* has 5 M and 4 S, and *F. pudica* has 1 M and 12 S.

The fragment chromosomes do not seem to affect the viability of pollen (*vide* Part IV) and they can be multiplied almost indefinitely (Darlington, 1930). They are therefore presumably inert, like the proximal parts of sex chromosomes in *Drosophila* and the B-type chromosomes in maize (McClintock, 1934).

I am indebted for the preparations to Mr. L. La Cour, who has elsewhere described the technique used (1931 and 1935). The fixations were made with medium Flemming or 2 BE, and the stain used was Newton's iodine-gentian-violet combination. The root-tips were sectioned at 40 μ , the pollen mother-cells and pollen smeared. I am also indebted to Mr. H. C. Osterstock for the photographs.

SUMMARY

1—In order to use comparative observations of chromosome behaviour for the inference of mechanical principles, it is necessary to define the assumptions justified by previous studies, combining them into a provisional axiomatic, and to test these assumptions by rigorously applying them to explain the observations. It is necessary to assume, *inter alia*, that two kinds of repulsion-centre exist in typical mitosis, the centrosomes or analogous bodies outside the nucleus, which determine spindle formation, and the centromeres inside the nucleus, which are specialized chromomeres and determine chromosome movement.

2—The group of organisms chosen for analysis is the genus *Fritillaria* (Liliaceae) in which behaviour is variable in respect of (1) polyploidy, (2) chromosome number and structural arrangement, including "fragmentation", (3) completeness of chromosome pairing in the prophase of meiosis, and consequently degree of localization of chiasmata at meiosis, (4) degree of contraction of the chromosomes at meiosis. These variations make it possible to infer the varying conditions of chromosome movement.

DESCRIPTION OF PLATE 10.

The first metaphase and anaphase of meiosis. *a*, *Fritillaria latifolia*, with extreme localization. *b*, *F. citrina*, with moderate localization. *c*, *F. obliqua*, with slight localization, showing two unpaired fragments and accessory plate formation. Microphotographs taken with 4 mm. objective and 15 eyepiece. $\times$ ca. 400.

The External Mechanics of the Chromosomes

II—MEIOSIS IN DIPLOIDS

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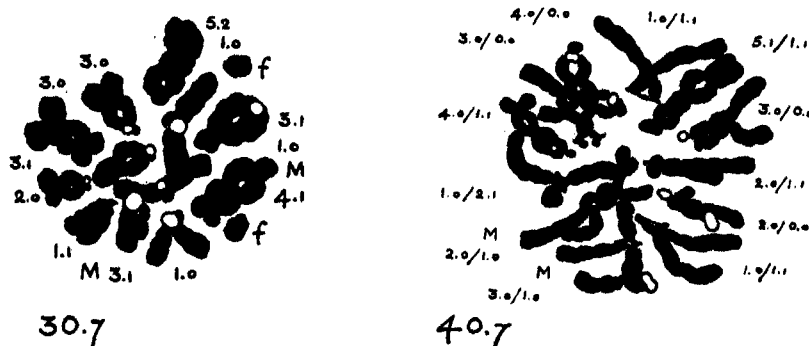
[PLATE 11]

1—INTRODUCTION: THE NORMAL AND THE UNIQUE

It has generally been believed that nuclear divisions, whether mitosis or meiosis, follow under average conditions in an average organism a certain course which is called "normal", for which one description may lastingly suffice. In regard to the prophase of meiosis it has become evident that this ideal normality merely represents a statistical mode and that every nuclear division witnesses its own combination of chances. The individuality of organisms is indeed anticipated, and determined, by the individuality of chromosome behaviour in the germ-cells of their parents. Behaviour is unstandardized and adaptation is imperfect. In considering the behaviour of the chromosomes at the metaphase of meiosis it is possible to carry this rediscovery of the unique a stage further. It is possible to show that in the metaphase orientation and anaphase disjunction of "normal" paired chromosomes in "normal" plants a variety of abnormalities actually occur. No two cells are alike, and in the analysis of their differences lies the means of finding out the forces at work.

2—PROPHASE MECHANICS: CHIASMA DISTRIBUTION AND MOVEMENT

The frequency of chiasmata can be studied between diplotene and metaphase in species with all degrees of localization, e.g., *F. imperialis* (Darlington, 1930), *F. Elwesii* (Darlington, 1935, a), and *F. meleagroides* (figs. 4 and 6, Table I), and *F. ruthenica* (Tables II and III). In all these species the number of chiasmata is constant at successive stages. There is no evidence that they ever fuse as a result of movement. The proportion that is terminal increases during the diplotene stage in the short arms of the chromosomes in all species, and in the long arms also in the



FIGS. 1 and 2—First metaphase of meiosis, fig. 1 in *F. obliqua*, fig. 2 in *F. acmopetala*. The numbers of total and terminal chiasmata given for each bivalent (in fig. 2 the two arms separately) and for all 12 bivalents below. M, the median bivalents. f, a fragment. Note—The detailed structure cannot be satisfactorily represented from polar views. $\times 1600$.

species without extreme localization, where some chiasmata have been formed close to the ends of these arms (Table III). This is due simply to the movement of the distal chiasmata to the ends.

Special configurations bear witness to the slowness of prophase

TABLE I—ANALYSIS OF CHIASMATA IN *F. meleagroides*, GIVING NUMBERS PER ARM-PAIR (cf. TABLE III)

2 nuclei each	2 M, each arm		10 S, long arm		10 S, short arm		Total per bivalent
	Total	Terminal	Total	Terminal	Total	Terminal	
Late diplotene	1.1	—	2.0	—	1.0	0.50	2.88
Diakinesis	1.5	—	1.85	—	1.0	0.85	2.88
Metaphase	1.4	—	1.75	—	1.0	0.95	2.76
Total	1.33	—	1.86	—	1.0	—	2.83

TABLE II—ANALYSIS OF CHIASMATA IN *F. ruthenica* (AT FIRST METAPHASE OF MEIOSIS (cf. TABLE III)

15 nuclei	Terminal chiasmata	Numbers of bivalents with respectively			Total chiasmata	Chiasmata per bivalent
		1	2	3		
		chiasmata per bivalent				
M type, 75 bivalents ..	0	27	47	1	124	1.66
S type, 60 bivalents ..	1*	12	44	2	—	—
	2	—	—	2	112	1.88
Total	—	—	—	—	236	1.77

* In short arm only.

movement. For example, chiasmata are not always terminalized in the short arms of chromosomes, where they are closest to the centromere (fig. 16). Again where there are few chiasmata they never reach an equilibrium position with equal loops such as is characteristic of the bivalents of *Stenobothrus*, *Hyacinthus*, *Agapanthus*, and other organisms with moderate chiasma-movement. Evidently, therefore, on the repulsion hypothesis of forces determining chiasma-movement, both the centromere-repulsions and the body-repulsions between chromosomes are very small in *Fritillaria*. Or, to be more precise, they are small in relation to the size of the chromosomes. The same repulsions which produce complete terminalization in *Oenothera*, *Primula*, and *Solanum* would not make a sensible change in the larger chromosomes of *Fritillaria*, even though the surface charge and the work done in repulsion were similar functions of size.

Localization of pairing and chiasma formation depend on very long chromosomes having time to pair only in their proximal parts (Darlington, 1935, *a*). It is therefore no coincidence that a minimum of terminalization is found in species with localization. Both are conditioned by the great size of the chromosomes.

With such slight chiasma movement we can attach more importance than usual to the distribution of chiasmata at metaphase. We can infer with confidence that their original positions were close to their final ones. Bivalents with extremely uneven distribution of chiasmata may therefore be taken to show the direct results of intermittent pairing, which is characteristic of all but the complete localization found in *Mecostethus*.

The variation in chiasma behaviour amongst species is not determined simply by different degrees of localization. The species vary independently in regard to chiasma frequency (Part I, Table I). This I have

shown already within *F. imperialis*, which has a free distribution of chiasmata (1930). Clonal varieties of this species have chiasma-frequencies ranging from 2.6 to 5.0 per bivalent. This being so the distribution and frequency of chiasmata must depend on two variable factors: length paired at pachytene and chiasma frequency per unit of this length.

As a measure of the degree of localization we cannot therefore take the chiasma frequency in particular proximal segments like the short



FIGS. 3 and 4—Early diakinesis with the bivalents drawn separately in *F. acmopetala* (fig. 3) and *F. meleagroides* (fig. 4). Nucleoli are shown as circles. The nucleolar chromosome in fig. 4 is interlocked with another. The chromosomes show the last traces of relational coiling. $\times 1600$.

arms of S chromosomes; we must take the proportion between this and the chiasma frequency in the longer segments. In this way it is possible to describe the degree of localization of a species quantitatively and arrange the species in order of degree of localization, as I have attempted to do in the table (Part I).

The double basis of variation in frequency is reflected in the statistical properties of chiasmata of whole nuclei, especially in species with intermediate degrees of localization which naturally have the most variable pachytene pairing. They have a greater variability than species with

TABLE III—COMPARISON OF CHIASMA FREQUENCIES AND POSITIONS IN SPECIES IN DECREASING ORDER OF LOCALIZATION (NUMBERS PER ARM-PAIR AND PER WHOLE BIVALENT, TERMINAL CHIASMATA IN BRACKETS)

Species	Nuclei	M, each arm	S, long arm	S, short arm	M : S	Mean per bivalent
1. <i>F. ruthenica</i>	15	0.84 (—)	0.88 (0.03)	1.00 (1.00)	1.68 : 1.88	1.77
3. <i>F. meleagroides</i>	6	1.33 (—)	1.86 (—)	1.00 (1.00)	2.66 : 2.86	2.83
7. <i>F. acmopetala</i>	4	1.56 (0.06)	2.17 (0.05)	0.80 (0.63)	3.12 : 2.97	3.00
12. <i>F. obliqua</i>	1	1.75 (0.50)	2.13 (0.30)	0.20 (0.20)	3.5 : 2.33	2.50

free distribution (cf. Darlington and Janaki-Ammal, 1932), as the following total number show:

Total Numbers of Chiasmata in Whole Nuclei

A—Species with extreme localization (cf. Table I, Part I):

1. *F. ruthenica* ($n = 9$): 12, 14(2), 15(2), 16(5), 17(4), 18.
2. *F. pontica* ($n = 12$): 20, 22, 23.
3. *F. meleagroides* ($n = 12$): 32, 34(4), 35.

B—Species with less extreme localization:

5. *F. Meleagris* ($n = 12$): 25, 26, 28, 29, 32.
7. *F. acmopetala* ($n = 12$): 23, 25(2), 33.
9. *F. citrina* ($n = 12$): 31, 34, 36, 37, 40.
10. *F. Elwesii* ($n = 12$): 32, 34, 35, 36, 37, 40.

Allowing for variations in chiasma frequency, we can define some of the special statistical properties of bivalents with localized chiasmata. First we see that in some species there is a higher chiasma frequency in the shorter S than in the longer M chromosomes. This property is peculiar to species with extreme localization such as *F. ruthenica*, *F. meleagroides*, and *F. pontica*. (The M chromosomes in the last species have the lowest chiasma frequency found in *Fritillaria*, and these alone I have seen, in one nucleus, as univalents at metaphase.) It is contrary to the rules observed in all species of plants and animals with complete pachytene pairing. For example, in *F. imperialis* with free chiasmata and even in *F. obliqua* with partial localization (Table III), M chromosomes have more chiasmata than S. It therefore clearly depends on the incompleteness of the pairing.

In order to understand this anomaly we have to call to mind how the prophase pairing takes place when it is incomplete. Pairing begins near the centromere and proceeds along the chromosomes until it is interrupted by their division. Those chromosomes which come together first near the centromere will therefore pair along the greatest length and form most chiasmata. Parts of chromosomes whose movements are hindered, such as those attached to nucleoli, do not always pair even in the "normal" species. Now it is clear that the *ends* of chromosomes will in these circumstances have an advantage in pairing over *middles*, being relatively unencumbered in their movements. Chromosomes with the centromeres near the ends will enjoy a special advantage over those with centromeres in the middle and will pair further. The S chromosomes will

therefore form more chiasmata than the M in species where the pairing is interrupted early and the localization is extreme.

It should follow, if this explanation is sound, that even the distal ends of long arms of chromosomes will have an advantage in pairing over the middle parts where pairing is incomplete. This indeed occurs. Occasional bivalents are found in both extreme and intermediate species having no chiasmata in the middle parts but a chiasma at the distal end, which is not merely remote from the other chiasmata but terminal. Since chiasma movement even near the centromere is slight, this chiasma must have been formed very close to the end (see fig. 12, and Newton and Darlington, 1930, fig. 7). The same kind of configuration has been found in *Mecostethus*, where the chiasmata are localized, by McClung (1928, *a, b*).

The advantage of ends in pairing also explains why many hybrids with defective pairing have such chromosomes as are paired associated by terminal chiasmata (e.g., *Triticum*, Darlington, 1930). The reason for failure of pairing in all such cases is, we must suppose, that the organisms are structural hybrids, i.e., their homologous parts are differently arranged in the pairing chromosomes. This is bound to delay their pairing and very often only the ends will have time to pair. The middle parts will divide before they can come together, or the association will be non-homologous, and chiasmata will be largely terminal. Structural hybrids will therefore often share one of the differential properties of organisms with incomplete precocity.

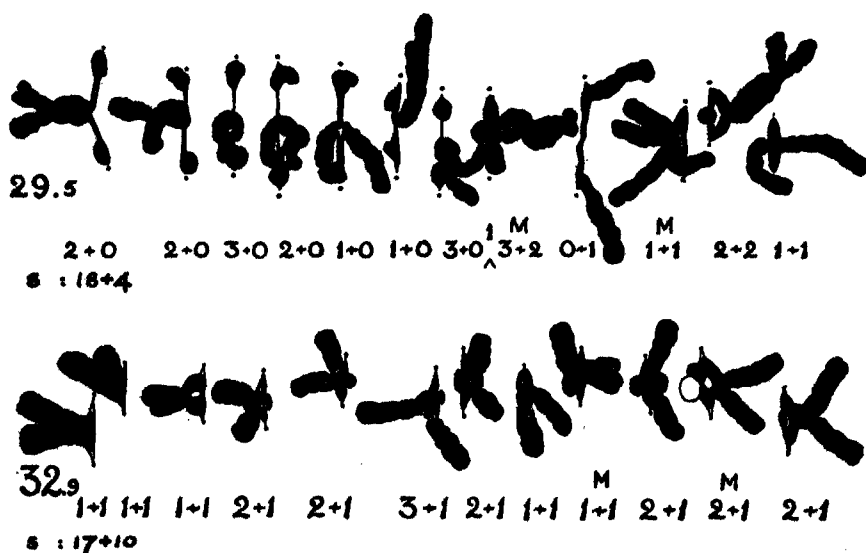
We may notice parenthetically that these observations indicate why large nucleoli are as a rule situated near the ends, the large nucleolar constriction separating the characteristic terminal satellite or trabant. Evidently they would hinder meiotic pairing too much if they lay in the middle of the chromosome. *Fritillaria*, *Lilium*, and *Hyacinthus* are exceptional in having large nucleolar constrictions one-third of the distance from their ends, but their chiasma frequency is well above the margin of safety for pairing. Even in *Fritillaria*, however, most of the nucleoli are sub-terminally attached.

It seems, therefore, that three position relationships govern the pairing of particular parts of chromosomes where the process is interrupted before it is complete: (i) their distance from the centromere, (ii) their distance from the ends of the chromosomes, (iii) their distance from a nucleolus, if one is attached.

3—ANAPHASE MECHANICS: THE CENTROMERE

The centromere has been illustrated at pachytene in meiosis (Janssens, 1924), in *Stenobothrus* (Darlington, 1932, *a*), in *Agapanthus* 1935, *a*, in

Fritillaria) and at somatic mitoses (Nawaschin, 1927, in *Galtonia*; Trankowsky, 1930, in various plants). It has been found at the meiotic prophase and metaphase in *Agapanthus*, *Zea Mays*, and *Tradescantia* (Darlington, 1933, *a*, 1934, *b*, 1936, *b*). In *Fritillaria* I have found it in faded gentian-violet preparations of first metaphase and anaphase, as a single brightly-staining particle attached to the two chromatids, lying towards the pole and away from the centromere of the partner chromosome as though repelling it. The two chromatids may be said to be fused at this point (Darlington, 1935, *a*). The relationships of chiasmata



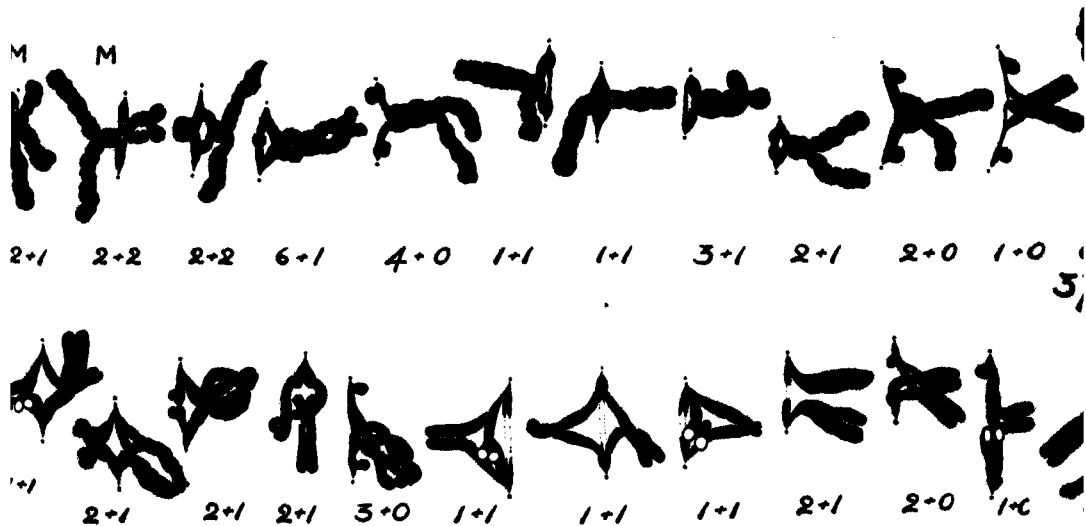
FIGS. 5 and 6—First metaphase in side view. The centromere is illustrated in fig. 6 (*F. meleagroides*). In fig. 5 (*F. hispanica*) it is inserted conventionally though invisible. Two chromosomes of *F. hispanica* have an interchange association. The numbers of chiasmata are given separately for long and short arms and the totals for the S chromosomes below. $\times 1600$.

on opposite sides of it cannot therefore be ascertained cytologically, even if they are already predetermined. As at pachytene, it seems to be about 0.25μ in diameter (fig. 7). Even in these large chromosomes it is therefore at the limit of visibility. I have also seen it once, less violently repelled from the main body of the chromosome, inside the nucleus at diakinesis (fig. 8) and at diplotene (fig. 26, Plate 11).

The threads connecting the centromere with the body of the chromosome on either side of it are what have often been called "spindle fibres", since the lines of weakness in the dessicated spindle with suitable fixation may be seen to continue their direction to the poles. They indicate that

a specially strong orientation of the spindle particles is determined by the reaction of the centromeres and the spindle-poles.

The singleness of the centromere at metaphase agrees with my expectation (Darlington, 1932, *a*, p. 343), based on the assumption that its division occurs only when it determines the separation of the two chromatids attached to it at mitosis and at the second division of meiosis. Its division at a later stage than the rest of the chromosome is evidence of its special chemical constitution, corroborating the evidence I have earlier adduced of its special surface charge. This charge is responsible for the localized repulsion which determines the anaphase separation and



FIGS. 7 and 8—First metaphase and anaphase in *F. acmopetala*, showing how the anaphase structure is related to the metaphase chiasmata, successive chiasmata being either compensating (third figure) or non-compensating (fifth figure). $\times 1600$.

in organisms with complete terminalization expands the proximal loops of bivalent chromosomes at the expense of the distal ones.

At anaphase of the first meiotic division the paired chromosomes move apart. They move as though they were drawn apart by the separation of the centromeres. At the same time a change takes place in the relationships of their paired chromatids. The attraction between them lapses. Their spatial dispositions are thus revealed and they are seen to be lying parallel except at the chiasmata, where they change partners. Where there are several chiasmata their relationships may be such as to give a multiple coiling of the chromatids (fig. 10). The lack of coiling at a

normal meiosis apart from chiasmata must be contrasted with the chromatid coiling at an abnormal meiosis in *Fritillaria* (Part V). The lapse in attraction must also be contrasted with its maintenance in *Hyacinthus* in the free arms of chromosomes in trivalents (Darlington, 1929, a).

The relationships of chiasmata, which are not usually clear until the lapse of chromatid attraction at anaphase, become clear in one special type of configurations at metaphase (figs. 10, 15, and fig. 24, Plate 11). In these configurations two cytologically compensating or "comparate" chiasmata (due to reciprocal or complementary crossings-over) lie very close together, and the paired chromatids are more widely separated than elsewhere, so that a gap appears between them, and it might be thought from seeing them in this one aspect that there were no chiasmata at all. In polar view a very small loop would, however, be visible between the chiasmata.

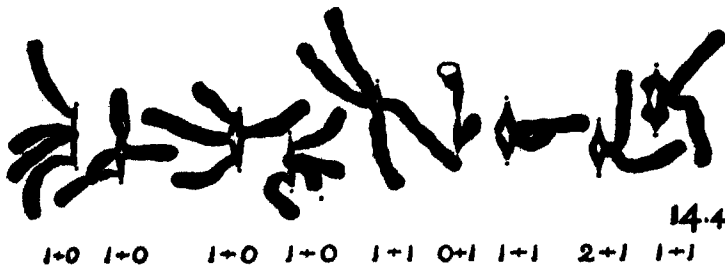


FIG. 9.—First metaphase in *F. ruthenica*, showing that the five longer M chromosomes have fewer chiasmata than the four shorter S chromosomes. $\times 1600$.

This abnormality provides the first cytological evidence of what on physico-chemical grounds we know must be the mechanical basis of chromatid pairing, viz., an equilibrium between forces of attraction and repulsion, and not an absolute and unqualified attraction, such as might be inferred from the typical appearance of contact. All relative positions of chemically similar bodies in the cell are, from analogy with inorganic mechanics, to be explained in terms of such equilibrium positions. The repulsion is evidently greater in proportion to the attraction when the related parts of the chromatids, between two chiasmata, are reduced in length; their equilibrium position is then further apart than normal and they can be seen not to be in contact. With non-compensating, "disparate" chiasmata the two pairs of chromatids on the distal side of the two chiasmata are not continuations of the two pairs on the proximal side, each of each. One pair is therefore relationally coiled and no gap is visible.

4—METAPHASE MECHANICS: ORIENTATION AND CONGRESSION

The arrangement of the separate chromosomes at metaphase of mitosis and meiosis may be considered in terms of the three following relationships:—

1. *Orientation* on the spindle, or the taking up of a direction related to the spindle arcs by the centromeres, whether they are polarized in a chromosome at mitosis, unpolarized in a univalent at meiosis, or related in pairs or larger numbers in bivalents or larger associations.

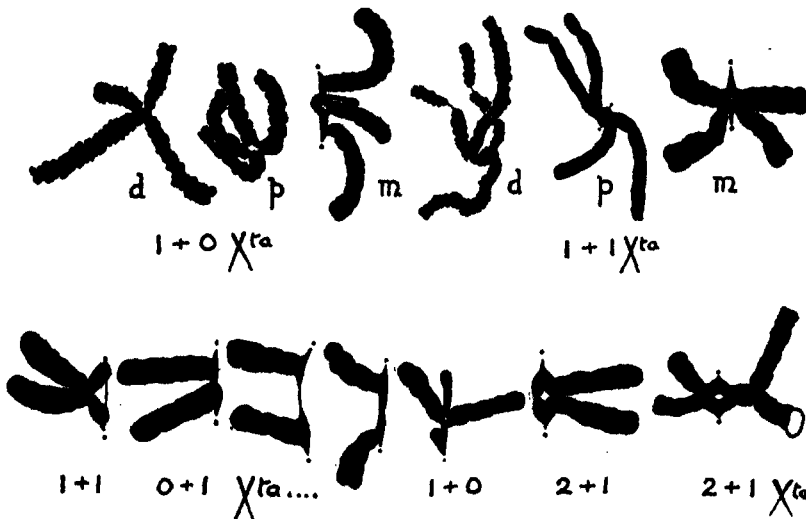


FIG. 10—Particular configurations from a clone of *F. ruthenica* with larger chromosomes. Above, left: M bivalent with a one chiasma at diakinesis, pro-metaphase and metaphase; right, M bivalent with 2 chiasmata at same stages. Note the nucleolar constriction at diakinesis. Below: bivalents of exceptional types. The numbers of chiasmata in long and short arms are given separately. The 2 + 1 S bivalent has two compensating chiasmata, and the gap between its chromatids is therefore visible from the side. Xta = chiasmata. $\times 1600$.

2. *Congression* on the metaphase plate, or the coming together of the chromosomes, symmetrically or otherwise, with regard to a plane equidistant from the two centrosomes or poles of the spindles.

3. *Distribution* on the metaphase plate, or the spatial arrangement of the separate chromosomes or configurations with regard to one another.

The typical distribution of the paired chromosomes on the metaphase plate follows the usual rules in *Fritillaria*. The centromeres lie on the spindle-arcs: arcs of circles passing through the two poles and symmetrical with regard to them. They are equidistant from the equatorial plane in the spindle. The different bivalents are more or less uniformly dis-

tributed in the equatorial region to form the "plate". This distribution may be explained as determined by the following conditions: (i) mutual repulsions between the spindle poles or centrosomes and the centromeres of all the chromosomes; (ii) a more efficient action of these forces along the spindle-arcs joining the two poles, analogous to the effect of a directionally differential dielectric coefficient, and determined itself primarily by the action of the centrosomes on the medium, and modified later by the action of the centromeres; (iii) a body-repulsion between all parts of all chromosomes and having a slighter effect than the other forces. The centromere-repulsion and body-repulsion correspond to the localized and generalized repulsions inferred from terminalization. The present observations provide particular evidence of certain of these assumptions by showing various departures from normal behaviour in

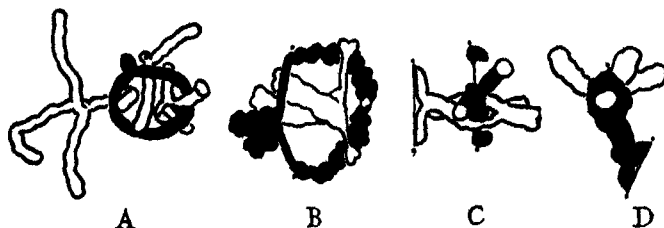


FIG. 11—Cases of interlocking. A, at diakinesis in *F. ruthenica* as a result of the exceptional formation of a distal terminal chiasma in an S bivalent. B, at metaphase in *F. latifolia*. C, in *F. hispanica*. D, in *F. pontica*, where the interlocking loop is so small that the bivalents are held in a rigid relationship, so that their spindle orientations consequently conflict but are none the less both effective in securing repulsion. $\times 1600$.

regard to orientation, congression, and distribution which may be traced to particular causes.

Where two bivalents are interlocked it may be impossible for both of them to lie with their centromeres on a spindle-arc. In the example illustrated (fig. 11, D), the displaced bivalent lies in a modified spindle-arc and shows the full degree of repulsion between its centromeres. This is not possible where the centromeres (with non-localized chiasmata) are further apart, as found by Dark in *Paeonia* (1936), Richardson in *Lilium* (1936), and myself (1936, a, b) in *Podophyllum versipelle*.

The diagonal repulsion is analogous to that between associated chromosomes in multiple rings of polyploids and structural hybrids. In such configurations one centromere may be seen in a mechanical relationship with two others passing to the opposite pole. Centromere repulsions need not therefore follow the predetermined arcs between the two centrosomes; these arcs can be modified to a limited extent, 10° to 15° ,

by the action of the centromeres themselves. Such a view is in accordance with the assumption that the centromeres and the centrosomes, being alike centres of repulsion, are alike capable of determining the orientation of particles in the cytoplasm, which constitutes the spindle. The lengthening of the spindle in hybrids or mutants where pairing has failed (Matsumoto, 1933; Dobzhansky, 1934; *et infra*) supports this assumption, for it shows that the broadening of the spindle depends on the presence of mutually repelling double centromeres, whether of bivalents or univalents, at the equator.

The body-repulsion is regularly shown by the behaviour of free arms, *i.e.*, arms of chromosomes on one side of the centromere without any chiasma. These lie towards the poles when the bivalent is in the centre of the plate, and away from the centre when it is on the edge of the plate (fig. 14). The same applies to the small fragment chromosomes (fig. 13).

In most organisms there seems to be ample room for the chromosomes to lie on the plate without this body-repulsion interfering with the symmetry of their arrangement. But in *Fritillaria*, owing to the great size of the chromosomes in relation to the cell and to the spindle, this is not always so. The body-repulsion becomes important. In the species with localized chiasmata, particularly, the chromosomes are long, their arms lie wide apart, and often, owing to there being only one chiasma between the main part of the chromosome and the centromere, this main part is constrained to lie in the equatorial plane (figs. 10, 11, and figs. 20, 22, Plate 11). It is not surprising, therefore, that, in the final equilibrium of the plate, bivalents are found here and there that are asymmetrically arranged. The simplest and commonest abnormality is that where a bivalent is lying with one centromere on one side of the plate while all the rest of the configuration is lying on the opposite side. The body-repulsions of the other chromosomes then stretch the connexion between one centromere and the proximal chiasma to make it longer than the corresponding part between the other centromere and the chiasma (fig. 14, and fig. 23, Plate 11). The two centromeres remain almost symmetrical with respect to the poles, thus showing that the tension in the chromosome is slight in effect as compared with the repulsion between centrosomes and centromeres.

A more serious abnormality arises when one or more bivalents fail to approach the plate at all and come to lie on one or both sides of it. This *non-congression* is found in the species with localized chiasmata, *F. ruthenica*, *F. citrina*, and *F. acmopetala*, where M chromosomes with a chiasma on either side of the centromere are affected, and also in *F. obliqua*, and *F. lanceolata*, with free chiasmata, where any chromosome

may be affected (Plate 10, Part I, and Plate 11). The bivalents lying off the plate are apparently repelled by those on the plate. They form an accessory plate, and it may be supposed (Koller and Darlington, 1934) that this is a secondary equilibrium position between the equator and the poles, and that the unpaired sex chromosomes in Orthoptera regularly, and the paired ones in Mammalia sometimes, take up this position on account of having a lower surface charge than the rest. In *Podophyllum versipelle* I have found that an unoriented bivalent is similarly excluded from the plate.

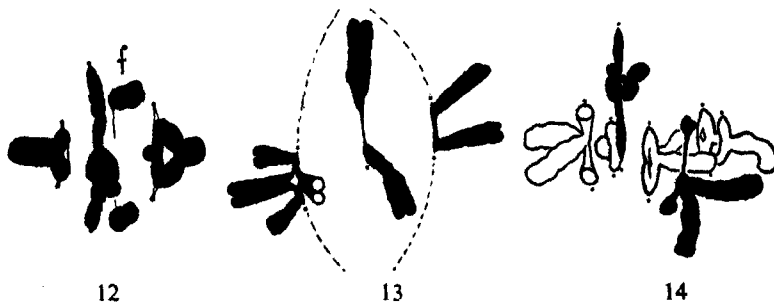


FIG. 12—Four bivalents, including a fragment bivalent with a chiasma in its short arm in *F. lanceolata*, with free chiasmata. The centromeres were visible. Note that their distance apart is governed by (i) the length of chromosome between them and the nearest chiasma. (ii) the length of chromosome between them and the second nearest, if it lies on the other side of them. FIG. 13—Arrangement of three M bivalents in *F. ruthenica*, showing that the positions of their free arms are determined by body repulsion, not by centrosome repulsions. FIG. 14—Asymmetrical bivalents produced by asymmetrical positions in *F. latifolia*. (Cf. fig. 23, Plate 11). $\times 1600$.

The effect of non-congression is that one of the daughter-chromosomes of the aberrant bivalent fails to fall in with the general mass of chromosomes passing to its pole. And failing to do so it is pushed back into the equatorial region by their body-repulsions and begins to behave as a lagging univalent whose fate may be traced through the succeeding stages (fig. 16). Such aberrant daughter bivalents might indeed be mistaken for chromosomes which had not been paired were it not for two special circumstances: in the first place the laggards were always single and their partners could be seen passing to one pole regularly and, in the second place, the excluded laggards occur in about 4% of the tetrads of the clone of *F. ruthenica* that I studied, although I never saw unpaired major chromosomes at the first metaphase in this or any other diploid species (except in the one case referred to in *F. pontica*).

The effect of the body repulsions on congression, or the coming together

of the chromosomes on the plate, depends on the chiasma structure, because this determines the space that a bivalent will take up on the plate. Only the M bivalents with a chiasma on each side of the centromere are forced off the plate in *F. ruthenica*. The bearing of this on the orientation of multivalents will be considered later.

Pairing therefore does not necessarily determine either orientation, or congression on the metaphase plate, or separation of the paired chromosomes to opposite poles, even with normal spindle formation. Where there are not enough pairs the spindle will fail to develop in its proper proportions; it will lengthen and bend in the way described by Matsumoto (1933) and Dobzhansky (1934), so that the pairs present will not behave normally. Where there are too many pairs they will not all succeed in

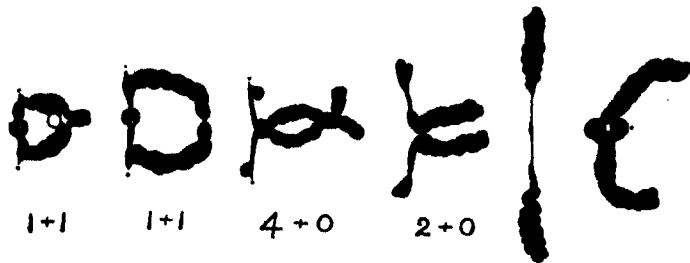


FIG. 15—Exceptional metaphase bivalents; the first three the result of intermittent pairing and chiasma-formation in *F. acmopetala*; the fourth a bivalent with compensating chiasmata in *F. latifolia* (cf. Plate 0, Part I); the fifth an S bivalent in *F. latifolia* with a chiasma in the short arm, an indication of extreme localization; the last a diakinesis S bivalent of *F. meleagroides* showing the centromere. $\times 1600$.

lying on one plate, and the aberrant pairs will fail to segregate equally. The spindle and the bivalents are therefore mutually adjusted parts of a mechanism which may go wrong through independent aberration in the one or the other; through a change in the number or form of the normally pairing bivalents as well as through the failure of the chromosomes to pair or of the spindle to develop at the right time.

SUMMARY

1—Fifteen out of twenty-four American and Eurasian species of *Fritillaria* have various degrees of *localization* of chiasmata in the proximal parts of chromosomes at meiosis.

2—These species vary also in chiasma-frequency between 1.8 and 3.0 per bivalent, but they all have the minimum of chiasma movement (*terminalization*).

3—The localization, being the result of interruption of pairing at zygotene, affects the chromosomes with median centromeres (M) encumbered with two long arms, more severely than those with sub-terminal ones (S) in which the pairing region is near a free end. The M

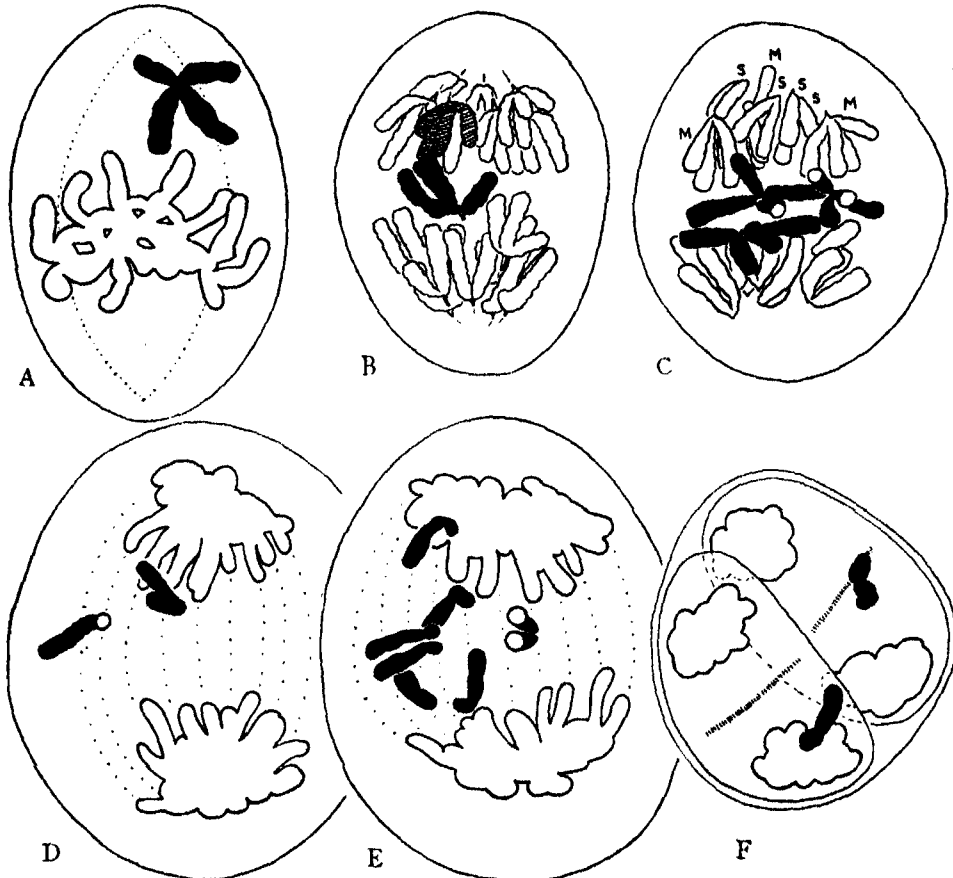
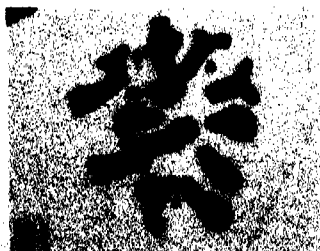
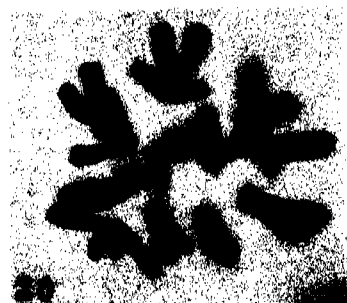
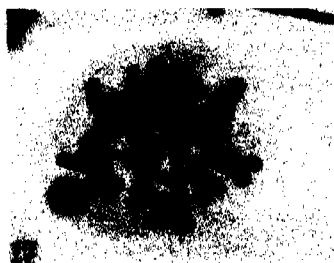


FIG. 16—The succession of events following accessory plate formation (non-congression) in *F. ruthenica*. A, metaphase. B, anaphase, one daughter bivalent lagging. C, later anaphase, three daughter bivalents lagging. D, telophase, two daughter laggards after division. E, telophase, four laggards dividing. F, second telophase, corresponding daughter univalents lagging in opposite cells. $\times 1600$

chromosomes therefore have fewer chiasmata than the shorter S chromosomes in the extreme cases of *F. ruthenica* and *F. meleagroides*.

4—The *centromere* (spindle fibre attachment chromomere) can be seen at pachytene, diplotene, diakinesis, metaphase, and anaphase in faded preparations. It is single, though the rest of the chromosome is double.





5—Interlocking modifies the *orientation* of the centromeres of bivalents. This orientation is therefore not determined absolutely by the centrosomes or spindle-poles but by a relationship between centromeres and centrosomes.

6—Owing to body-repulsions, the *congression* of the chromosomes on a single first metaphase plate at meiosis is conditioned by the presence of the right number of bivalents, for in many species, owing to crowding, bivalents may form accessory plates and their daughter halves may then behave as univalents and divide at the first division.

7—Thus irregular congression, as well as occasional failure of pairing due to absence of chiasma-formation, may occasion unequal segregation ("non-disjunction") in homozygous organisms typical of their species.

DESCRIPTION OF PLATE 11

First metaphase of meiosis in diploid forms.

FIG. 17—Typical pollen mother-cell of *F. pluriflora*.

FIGS. 18 and 19—Abnormal cell (*cf.* Part V). Fragment at 6 o'clock.

FIG. 20—*F. latifolia*; 2 M bivalents at 12 and 7 o'clock with all arms pushed outwards.

FIG. 21—*F. imperialis*; 12 fragments (*cf.* Darlington, 1930).

FIG. 22—*F. ruthenica*; M chromosome with 2 chiasmata lying off the plate (*cf.* fig. 16).

FIG. 23—*F. latifolia*; asymmetrical bivalent (*cf.* fig. 14).

FIG. 24—*F. latifolia*; bivalent with two compensating chiasmata close together (*cf.* fig. 15).

FIG. 25—*F. gracilis*; beginning of anaphase, showing separation of chromatids beginning.

FIG. 26—*F. Elwesii*. Diplotene nucleus, twice re-stained and re-mounted. The two centromeres of an M bivalent are shown at 8 o'clock lying between the usual two chiasmata.

Microphotographs taken with 1.5 mm. objective and 10 eyepiece. $\times 1500-2000$. F., two corresponding daughter laggards, lagging at the end telophase.

The External Mechanics of the Chromosomes

III—MEIOSIS IN TRIPLOIDS

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(Communicated by Sir Daniel Hall, F.R.S.—Received 15 April, 1936)

[PLATE 12]

I—INTRODUCTION

Triploid organisms have three homologous chromosomes of each kind instead of the two of diploids. The regular mechanism of heredity fails in these circumstances. The triploid is incapable of breeding true by sexual reproduction. But the way in which it carries out the process of chromosome pairing and segregation is of great significance. The processes take place in normal series, but the relationships they establish are abnormal. A triploid thus provides a natural experiment, with the diploid of its own species as a control for one variable, and with triploids of different species as controls for others.

In *Tulipa* and *Hyacinthus* I have made use of this experiment for inducing the principles of the external mechanics of chromosomes during the prophase of meiosis. I have inferred from them the relationships between the forces working in mitosis and meiosis. The triploid forms of various *Fritillaria* species make it possible to test the principles of metaphase mechanics induced from observations on structural hybrids and other polyploids (Darlington, 1932, *b*, and 1933, *c*) as well as from the exceptional behaviour in the diploid species of *Fritillaria* already discussed.

The chief source of material is a triploid form of *F. lanceolata* from California. It is an auto-triploid arising without hybridization, so far as its chromosome behaviour shows. The diploid of the species has a regular meiosis in the pollen mother-cells resembling that of *F. imperialis* (Darlington, 1931, *a, b, c*) with the 10 S and 2 M chromosomes characteristic of the genus, and with four or five chiasmata per bivalent freely distributed along the chromosomes (fig. 12, Part II). The diploid plant that I studied also had two homologous fragments like some clones of *F. imperialis*. These are not present in the triploid.

Three other triploids that I have examined, *F. pudica*, *F. recurva*, and *F. dasyphylla*, are similar in behaviour and are also derived from diploids

with free chiasmata. A fourth, *F. latifolia major*, is derived from a diploid with localized chiasmata; I have seen only its second divisions.

2—STRUCTURE OF TRIVALENTS: CHIASMATA

The thirty-six chromosomes appear usually as 12 trivalents at the first metaphase of meiosis in the pollen mother-cells. Occasionally one or even two trivalents are replaced by bivalents and univalents. The high frequency of trivalents as compared with triploid *Tulipa* is expected in view of the higher chiasma-frequency. The average frequency per set of three approaches six (fig. 1) and, like that of all other auto-triploids

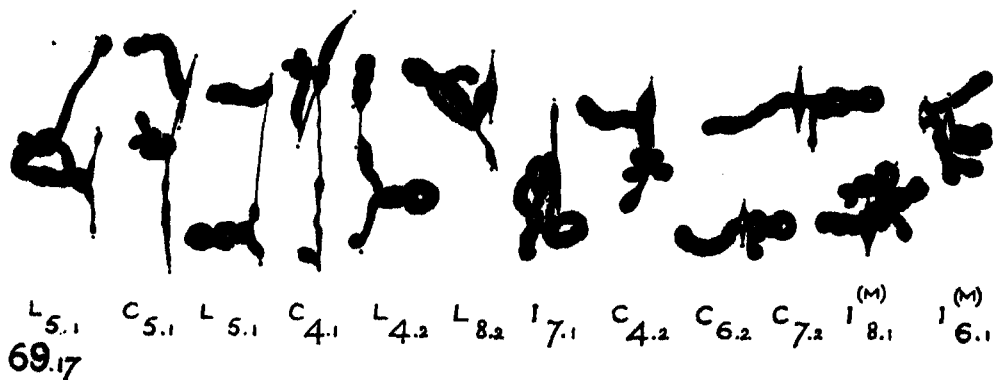


FIG. 1.—The twelve trivalents, in side view, of a first metaphase in a pollen mother-cell of *F. lanceolata*. The dot represents the usually invisible centromere of each chromosome. Where this lies over or in a chromosome its position has been shown by a dot at the side. M, the chromosomes with median centromeres. The figure give the numbers of total and terminal chiasmata. L, linear arrangement; C, convergent; I, indifferent. Note the linear arrangement is in an arc. $\times 1600$.

(including *Drosophila*, to judge from crossing-over data), exceeds the frequency per bivalent in the corresponding diploid; indeed, it exceeds that in any diploid *Fritillaria*.

The triploid *F. pudica* has a lower chiasma frequency (fig. 4) and the higher frequency of univalents correlated with it. As many as four were found in one nucleus. In this triploid I have illustrated the association in pairs at the pachytene stage (1935, a). This takes place with occasional exchanges of partner, one chromosome being always unpaired at any particular position in the linear thread, as in *Tulipa*, *Hyacinthus*, and *Zea*.

The chiasmata, as in the diploid, are freely distributed along the chromosomes. Their distribution among the possible pairs is also free, subject to a limitation in the number of changes of partner at pachytene con-

sistent with the view that there are a certain limited number of "pairing blocks" at this stage. The effect of this limitation has been shown from an analysis of variance in chiasma frequency in triploid *Tulipa* (Darlington and Mather, 1932). It may be seen equally from the comparison of the frequency of particular kinds of trivalents in *Fritillaria* owing to the higher number of chiasmata. Thus it will be noticed that, while many

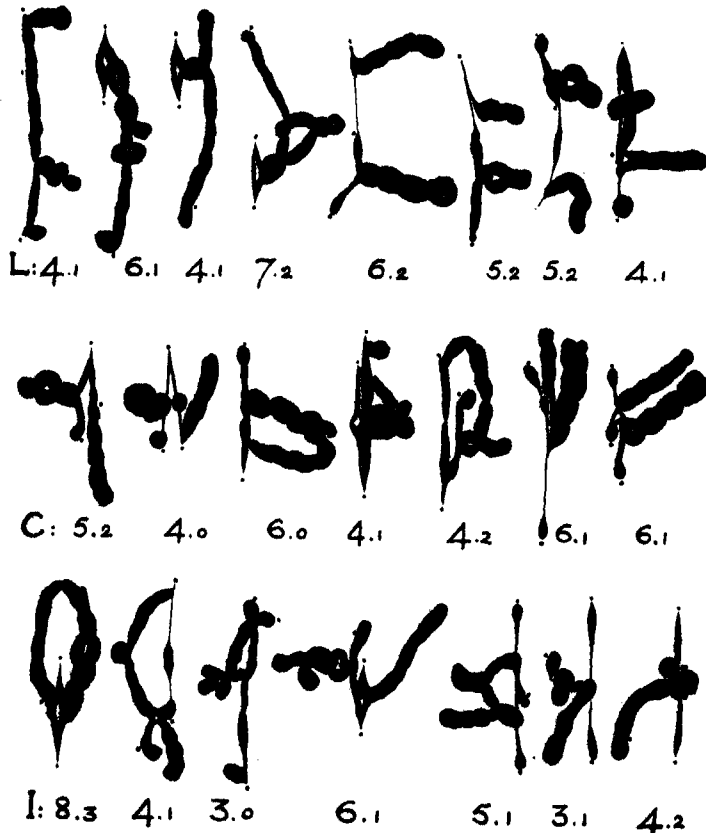


FIG. 2.—Trivalents of S chromosomes (with sub-terminal centromere) in *F. lanceolata* grouped according to the type of arrangement on the spindle (L, C, and I) with numbers of chiasmata in each configuration. $\times 1600$.

trivalents have four chiasmata in succession between two of the chromosomes, no trivalents have four chiasmata in succession between different pairs of chromosomes (figs. 1-4), an event which should be at least as frequent in the absence of a limitation on exchanges of partner at pachytene.

One of the three centromeres of a trivalent is usually far apart from the other two, and their arrangement is always asymmetrical owing to the

asymmetry of the trivalent. The formation of a metaphase plate comparable with that in a diploid is therefore out of the question. The great bulk of the spindle is filled with chromosomes stretching almost to the poles.

The arrangement of the individual trivalents may now be considered in detail.

3—METAPHASE ORIENTATION

The one constant property of the arrangement of the trivalents is that their three centromeres lie on the spindle. Their average distances from



FIG. 3—Trivalents of M chromosomes in *F. lanceolata* with linear and indifferent arrangements. $\times 1600$.

the two poles are approximately equal in the different trivalents as a rule, but not always. The bodies of the chromosomes may lie off the spindle. The spindle-pole determinants and the centromeres are therefore of primary importance in arrangement, the bodies secondary.

Let us consider first the two centromeres of the three which are closest together. These are the centromeres of the chromosomes which have a chiasma closer to the centromere than the third has. The pair usually lies, together with the stretched part of the chromosomes proximal to the first chiasma, in a particular relationship with the spindle which can be seen in side view most clearly when the chiasma is not very close to

the centromeres. They lie on arcs of circles passing through the poles; these spindle arcs are necessarily more concave near the edge of the spindle. The spindle may be taken as made up of an indefinite number of such arc-axes and I shall speak of their direction as axial.

With reference to this simple or dominant arrangement shown by pairs of centromeres, the various arrangements of threes can be classified as of three types:

- (1) *Linear*: all three centromeres lie in the same axis.
- (2) *Convergent*: two lie in different axes which converge on the third. These axes are evidently modified axes, as are those of interlocked bivalents.
- (3) *Indifferent*: one of the centromeres shows no axial relationship with the other two and the chromatid connecting it with its proximal

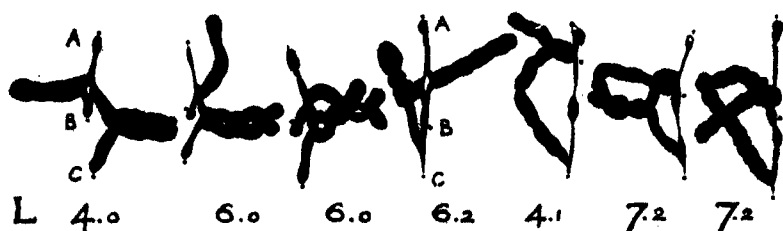


FIG. 4—Asymmetrical tensions in proximal segments (between centromere and first chiasma) of S type trivalents in *F. lanceolata* with linear arrangements. $\times 1600$.

chiasma shows no tension. This centromere has been twice seen to divide at metaphase (fig. 1, M), and its later behaviour may certainly be that of a univalent, for laggards are often found dividing at the first division, as in triploid *Hyacinthus* where univalents are also rare at metaphase.

A fourth and fifth system of *parallel* and *discordant* arrangements occur only with four or more chromosomes and will be considered later.

Many variable factors seem to contribute to determining which of the three types the arrangement shall follow. We already know that all three may be taken in different nuclei by the same group of chromosomes in a ring of *Oenothera* or *Rhoeo*, in which the chromosomes themselves, and their associations by terminal chiasmata, are identical. Evidently, therefore, the chances of position—the order of establishing a relationship with the spindle—affect the result (Darlington, 1931, c). But it is necessary to suppose that the chiasma structure also affects the chance of assuming a particular arrangement. As I have pointed out (1931, c), interstitial chiasmata give a greater rigidity to a multiple configuration and determine

a higher amount of non-disjunction in *Pisum* and *Zea* than in *Oenothera*. These *Fritillaria* trivalents show how the particular positions of chiasmata influence the result. Thus, in the M trivalents, convergent or indifferent arrangements arise only when one of the centromeres lies in a free arm, i.e., having chiasmata only on one side of it. Further, convergent arrangements never arise in S or M chromosomes except where two of the centromeres are approximately equidistant from the third and relatively close to their proximal chiasmata. These conditions are both rare in the M trivalents and convergent arrangements have never been found in them.

4—ANAPHASE DISJUNCTION

All convergent trivalents presumably divide so that two chromosomes go to one pole and one to the other. Most of the linear trivalents probably do the same, for the association of the middle chromosome in a linear arrangement is usually so much more extensive with one of its partners than with the other that it will readily be carried towards one pole without active repulsion of its own centromere. The same applies also to most of the indifferent arrangements. In some of the linear and indifferent types, however, one chromosome is evidently left lying on the equator to split like a univalent at the first division. This is shown, as in triploid *Hyacinthus* by laggards being frequent at anaphase, although univalents are rare at metaphase.

Laggards are infrequent in all four triploids with free chiasmata, however, and the later course of meiosis is generally regular. Restitution nuclei are never formed. In *F. latifolia major*, on the other hand, restitution nuclei are formed in every cell in some anthers. This is probably due to a high frequency of univalents resulting from localization of chiasmata, and a consequent reduction in the number of prophase pairing blocks. The chromosomes appear, then, at the beginning of the second division in a crescent-shaped body, the result presumably of stretching and bending of the first-division spindle (Plate 12).

5—STRUCTURAL HYBRIDITY

In diploid forms of three species, *F. latifolia*, *F. Elwesii*, and *F. pontica*, I have found occasional evidences of structural hybridity, in the shape of associations between four chromosomes resulting from translocation or interchange (fig. 5). In all the triploid forms studied, such abnormalities occur, and much more commonly. This is expected, since one chromosome of each set in the triploid is unpaired and small segments are therefore

more likely to find their translocated and reduplicated homologous partners. As a result of such structural changes, associations of six or of four and two replace the normal trivalents (figs. 5 and 6).

These larger configurations have repulsion arrangements in keeping with those of trivalents, but more complex. Thus there are not only simple linear arrangements but also two or three *parallel* ones, and com-

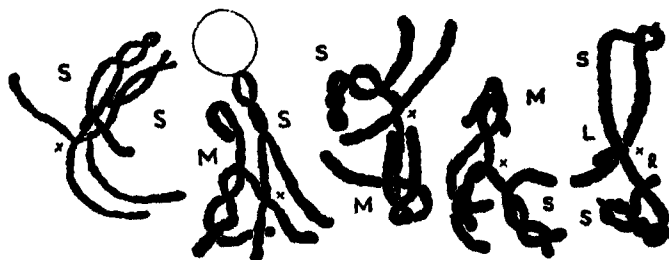


FIG. 5—Associations of 4 chromosomes (M and S type) at diplotene in diploid *F. Elwesii*. The chiasma between relatively translocated segments is marked with a cross. On the left such a chiasma is between chromosomes showing left and right relational coiling. $\times 1600$.

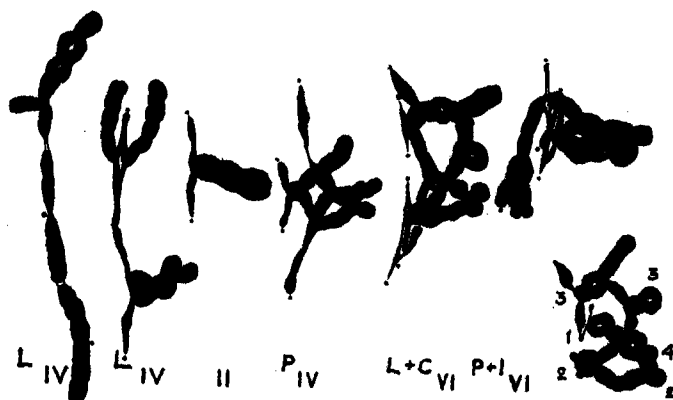


FIG. 6—Associations of 6 chromosomes (or four and two), S type, at first metaphase in triploid *F. lanceolata* (in side view, except the rightmost, for which numbers of chiasmata are given). Types of arrangement: P, parallel; C, L, and I as before. $\times 1600$.

binations of linear with convergent, parallel with indifferent and parallel with convergent; there are also multiple parallel arrangements. With small chromosomes as many as five centromeres may lie in one row (cf. Darlington and Moffett, 1930, on polyploid *Pyrus*).

Frankel (unpublished) has found the formation of chiasmata between relatively inverted segments in *F. dasyphylla* ($2x$ and $3x$), and I have found it in *F. pallidiflora*. It may be mentioned that its frequency is likewise

greater in triploid than in diploid forms, and indicates likewise that the dislocated segments are present as reduplications which have the chance of meeting only in the odd set of the triploid. It might be assumed alternatively that the triploids are more hybrid than the diploids, but in the absence of tetraploid species, and equally of triploid species independent of diploids, this is unlikely.

6—ANALYSIS OF FORCES

Comparison of the different types of trivalent reveals the forces at work in arranging them on the spindle.

Asymmetrical dispositions of two centromeres which are themselves structurally symmetrical with regard to the proximal chiasma arise through the presence of the third centromere in three ways.

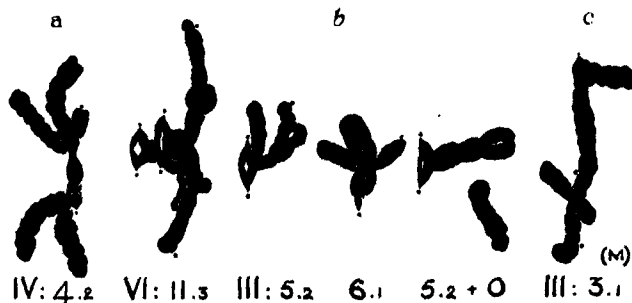


FIG. 7—(a) Association of four from diploid *F. latifolia*; (b) association of six and trivalents from triploid *F. recurva*; (c) M trivalent from triploid *F. pudica*. $\times 1600$.

The simplest is where the three centromeres (A, B, C) are linearly arranged, and their chromosomes are associated by chiasmata all on the same side of them (fig. 4, first three configurations). The arm with the middle centromere B would then be as long as that with A if it were not for the action of C on B which shortens its arm.

The second type, also linear, is that where the centromeres B and C lie within a closed loop owing to having chiasmata on both sides of them (fig. 4, last four configurations). The longitudinal cohesion of the chromosome parts within this loop prevents an extension of the arm of B within the loop equal to that of the free arm of A corresponding to it.

The third type is one with convergent arrangement of the three centromeres lying, however, in free arms (fig. 2, C, No. 6). The arm of one is then bearing a tension equal to that borne by the other two and is consequently more extended.

In the first of these types we have free arms of chromosomes that are structurally symmetrical showing different degrees of tension as a result of the action of the third centromere. Configurations of this kind prove that the centromeres act on one another; there is a relationship not merely between centrosome (or spindle pole) and centromere, as has been generally believed, but also a relation between centromere and centromere. Moreover, both relationships must be described as repulsions. This is a more precise statement of Belar's conclusion (1929, *a, b*, 1933) that the anaphase movement of chromosomes was "teilweise autonom", although it was, of course, impossible for Belar to assume that such bodies as centromeres constantly repelled one another, since homologous chromosomes were at that time supposed to attract one another in an undefined way.

When we apply the assumption of repulsion between centromeres to the analysis of the configurations we see that they show the expected inverse relationship of effect and distance. When with linear arrangement (fig. 2, L) two of the three are held closer together than either of them is to the third, the chromatids joining them to the nearest chiasma are more stretched than the longer part joining the third to its nearest chiasma. And when this longer part includes most of the chromosome it fails to show the expected tension due to repulsion, and, although having a linear arrangement, lies in the relaxed attitude of a chromosome with indifferent arrangement (fig. 3). Were the poles or centrosomes not concerned, the centromeres would always show tension between their centromeres. Complete relaxation of orientated chromosomes therefore shows that the position of the two or three linearly arranged centromeres is determined by the equilibrium of their repulsions for one another and for the two centrosomes.

The equilibrium position is modified by the tension on the chromosomes between the centromeres and their associating chiasmata, where the chiasmata are close. Where the chiasmata are remote this tension has no effect, and we then see that the repulsion exerted by the centromeres at metaphase is about equal to that exerted by the centrosomes. When free to do so, the centromeres lie as near to the poles as to one another.

In a word, the chiasmata in trivalents make a natural experiment, or rather a whole series of natural experiments, for us, showing how centromeres connected in different ways react with the spindle and with one another. But the varying behaviour of trivalents reveals more than this. It shows that the repulsions of the centromeres have a secondary effect. With the convergent arrangement they modify the spindle axes, and further, this modification is associated with a repulsion between the

centromeres just as the original formation of the spindle is associated with a repulsion between the centrosomes.

Evidence of modification of the spindle, due to the centromeres behaving like spindle poles, may be found in the behaviour of the lagging univalents, which often get a cell-wall formed between themselves and the main nuclei (Part V, fig. 3, and Darlington, 1929, *a, b*, figs. 39 to 41). In living cells of plants with this type of division the spindle can be seen to be actively related to the formation of the new cell wall (Belar, 1929, *b*). The abnormal cell wall must therefore be determined by an abnormal spindle modified by the centromere of the univalent. It cannot be due to the cell wall not being able to cut through a chromosome, for this has been frequently illustrated (*cf.* Darlington, 1929, *a*, fig. 42). Furthermore, it must be due to the centromere, for acentric chromosomes formed by crossing-over in inversions never determine the formation of cell-walls (Frankel, *unpublished*).

This conclusion leads us to a new view of the relationship of the centromeres (or the repulsion centres of the chromosomes) and the centrosomes (or the organs determining the formation of the spindle). I have earlier inferred (1932, *a, b*) that the centrosomes and centromeres have an analogous effect in setting up repulsion, the centrosome repulsion determining the metaphase plate, the centromere repulsion determining terminalization. If they also have an analogous effect in determining the orientation of the spindle it is worth while enquiring whether the analogy in their behaviour cannot be carried further. We find, in fact, a similarity in many respects. Their staining reaction is fugitive; their division-cycle is usually correlated with that of the chromosomes and their effect on cell-plate development is analogous; they are, so far as we know, of similar dimensions, being probably ultra-microscopic in many organisms. It is therefore possible that they are similar in their innate properties, and that the differences in their behaviour are due to the difference in their position in the cell.

7—SUMMARY

1—The chromosomes usually form 12 trivalents in the pollen mother-cells of triploids of species of *Fritillaria* with free chiasmata. Owing to interchange or translocation, associations of six are occasionally formed.

2—Owing to the association of chromosomes in *threes* being constituted by exchanges of partner between *pairs* of chromatids (at chiasmata), they are never symmetrical. The properties of a symmetrical bivalent may be explained in terms of the relationship of each centromere to the

poles. They are consistent with the spindle fibre theory of attraction. The asymmetrical trivalent shows that this is not sufficient: the relationships of the chromosomes themselves are important in two ways: (i) the centromeres modify the direction in which the spindle is differentiated, so that *convergent* as well as *linear* and *indifferent* relationships arise; (ii) The state of tension of the chromosomes between the centromeres and the nearest chiasma is an inverse function of the distance of the centromeres apart, provided that this distance is not greater than that at which equilibrium is reached with the polar repulsions.

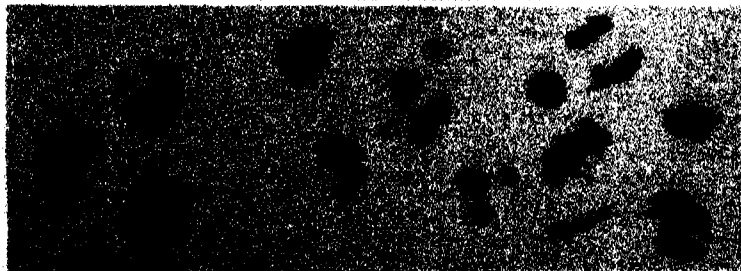
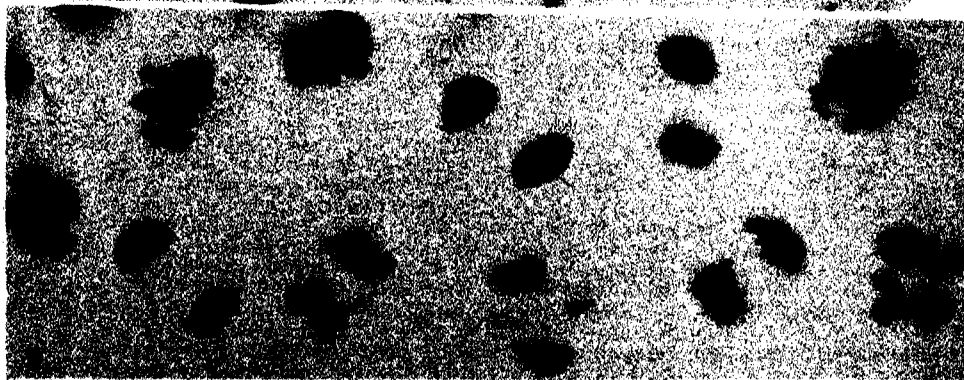
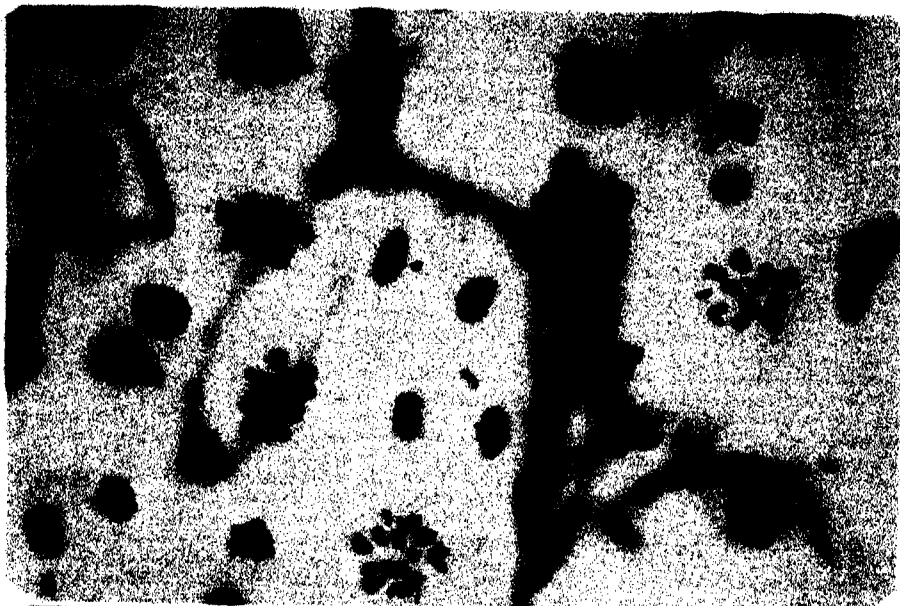
3—The centromeres of each chromosome configuration lie in an equilibrium position at metaphase, determined by their reciprocal repulsions and those of the poles, and modified by the tension of the chromosomes. At metaphase as well as at prophase the centromeres of the chromosomes therefore repel one another. In doing so they modify the structural orientation of the spindle, and at anaphase they control the spindle, which changes its shape and becomes a centromere spindle. The centromeres are in these and in other ways analogous to the centrosomes, and for this reason I have adopted the term centromere to describe them in place of the many compound expressions used in the past.

DESCRIPTION OF PLATE 12

Meiosis in triploid forms

FIGS. 8 and 9—*F. dasyphylla*, showing univalents and trivalents at first metaphase and some lagging univalents at first anaphase and telophase. $\times 400$. FIG. 10—Second metaphase in *F. latifolia*, all crescent shaped, and derived from restitution nuclei, and two second telophases formed without restitution. $\times 300$.

(Microphotographs taken with 4 mm. objective and $\times 15$ eyepiece.)



The External Mechanics of the Chromosomes

IV—ABNORMAL MITOSIS AND MEIOSIS

By C. D. DARLINGTON, John Innes Horticultural Institution

(Communicated by Sir Daniel Hall, F.R.S.—Received 15 April, 1936)

A—MITOSIS IN POLLEN GRAINS

[PLATE 13]

1—INTRODUCTION: THE INDIVIDUALITY OF POLLEN GRAINS

The nuclear divisions in the pollen grains are of special importance because each pollen grain is a genetical individual genetically distinct from all others. A study of pollen grain behaviour is therefore a genetical experiment of a simple kind. In the pollen grains of a hybrid we can not only distinguish the individual segregates which differ in the form and number of their chromosomes, but we can also compare their viability. We may even find differences in the mechanical behaviour of the chromosomes in the segregates, depending on their own genetic differences or conditioned by the variable reaction of the genotype of the parent plant. Such differences usually cripple or kill the organism and are therefore never available for study in somatic mitosis; we know of none that is compatible with survival. An apparent exception, Beadle's (1932) "sticky chromosome" mutant in maize, probably owes its character merely to a high rate of structural change. We may, on the other hand, expect to find them in the sexually specialized cells such as pollen grains, especially in species whose methods of propagation enable them to dispense with sexual reproduction. Beadle (1931, 1933, *b*) has discovered a number of such genetic abnormalities in pollen grains, abnormalities of great theoretical significance.

The present study is an attempt to compare the pollen grains of pure forms and segregating hybrids in *Fritillaria* from the mechanical as well as the genetical standpoint. The plants used are hybrid, some in respect of having fragments and others in respect of having a supernumerary set of chromosomes. The following species were studied (*cf.* Part I):

Diploid:

<i>F. ruthenica</i>	$x = 9$
<i>F. aurea</i>	$x = 12$
<i>F. recurva</i>	$x = 12$
<i>F. Eggeri</i>	$x = 12$
<i>F. askabadensis</i>	$x = 12$
<i>F. libanotica</i>	$x = 12$
<i>F. macrandra</i>	$x = 12$

Triploid:

<i>F. lanceolata</i>	$x = 12$
<i>F. pudica</i>	$x = 13$

Diploids with fragments:

<i>F. lanceolata</i>	$x = 12$
<i>F. imperialis</i>	$x = 12$

2—DIFFERENTIAL VIABILITY

The pollen grain mitoses in the anthers of the diploid species show the normal reduced numbers of chromosomes (figs. 3 and 4). Varieties of *F. imperialis* having fragments were studied in order to find out whether the number of fragments influenced the survival of the pollen grain. The results with "Orange Brilliant" having one fragment can be taken to correspond directly with the results of meiosis, and thus indicate an equal chance of survival, for a large proportion of fragments are lost at meiosis. A similar proportion with fragments is found in "Crown upon Crown", which normally has three in the parental somatic nuclei. Probably the



FIG. 1—Root tip mitosis in diploid *Fritillaria pudica*; $2x = 26$. 2 M-chromosomes, 24 S-chromosomes. S-chromosomes with longer short arm than the rest are marked S. Chromosomes spaced. $\times 1800$. FIG. 2—The same in the triploid form; $3x = 39$. $\times 1800$.

flower examined had lost two of its fragments somatically. Selective observation, owing to different rates of development, could not be responsible, for the data from several preparations were consistent (Table I).

TABLE I—DISTRIBUTION OF 80 POLLEN GRAINS WITH RESPECT TO NUMBER OF FRAGMENTS (*ff*) IN VARIETIES OF *Fritillaria imperialis*

Variety	Parental complement	Pollen grains		
		12	$12 + f$	Total
Orange Brilliant	$24 + f$	34	13	47
Crown upon Crown	$24 + 3ff$	26	7	33

The pollen grains of triploid *Hyacinthus* and *Tradescantia* show the frequency of extra chromosomes expected from the observation of meiosis, *i.e.*, with a binomial distribution modified by some loss of lagging

chromosomes (Darlington, 1932, *a*, Table XXXVIII). The pollen grains of diploid *Fritillaria* species, on the other hand, show a great excess of those with numbers approaching the lower extreme, the basic number, which as I have pointed out (1929, *a*) should be the most nearly balanced (Table II). Further evidence of the individual properties of the pollen grains appears from the fact that the three preparations of *F. lanceolata* showed divisions of pollen grains with different numbers (Table III), thus indicating that these have different rates of development. The haploid,

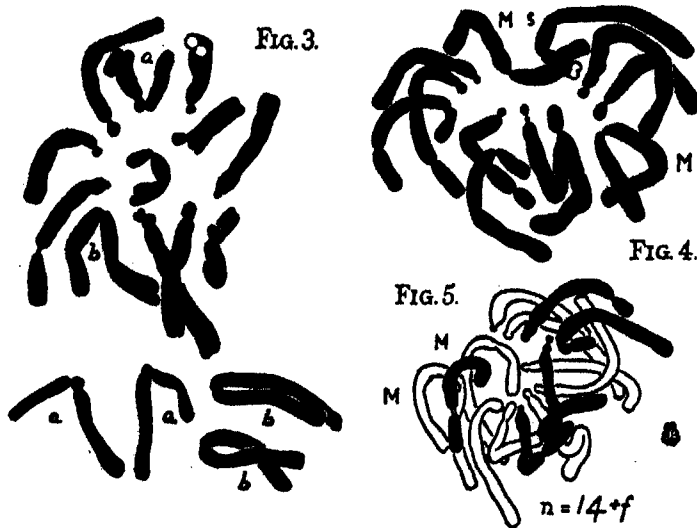


FIG. 3—Pollen grain mitosis in *Fritillaria askabadensis*; $n = 12$. *a* and *b*, two types of M chromosomes, drawn below separately from root-tip mitoses. $\times 1800$. FIG. 4—The same in diploid *F. recurva*, $n = 12$. *S*, a subterminal type resembling the *a* of *F. askabadensis*. $\times 1800$. FIG. 5—The same of triploid *F. lanceolata*; $3x = 36$, $n = 14 + f$. Types of chromosomes with secondary (nucleolar) constrictions shown black. Fragment abnormal, probably owing to its having formed a separate nucleus. $\times 1800$.

which appears as frequently in *Hyacinthus* as it should on a random expectation, appears here once in three pollen grains instead of once in 4096 (2^{12}).

TABLE II—DISTRIBUTION OF 21 POLLEN GRAINS OF TRIPLOID *F. pudica* WITH RESPECT TO THE NUMBER OF CHROMOSOMES ($x = 13$)

Chromosomes	15	16	17	18	19	20	21	22	23	24	25
Pollen grains	4	4	4	4	2	2	1	1	—	—	1

TABLE III—DISTRIBUTION OF 9 POLLEN GRAINS OF TRIPLOID *F. lanceolata* WITH RESPECT TO THE NUMBER OF CHROMOSOMES ($x = 12$)

Chromosomes	12	13	14	15	16	17	18	19	20
Pollen grains—									
First anther	3	—	—	—	—	—	—	—	—
Second anther	—	—	1	1	—	—	—	—	—
Third anther	—	—	—	—	—	1	—	1	2

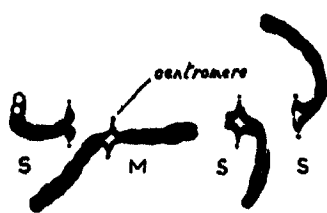


FIG. 6.

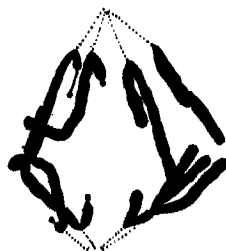


FIG. 7.

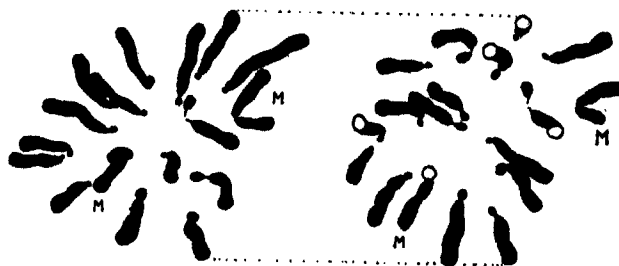
 $n = 17$

FIG. 8.

FIG. 6—Four chromosomes in side view in pollen grain mitosis of *F. libanotica* ($n = 12$). The centromere of the M chromosome was stained and visible. Its position, though invisible, is indicated in the other S. FIG. 7—Four chromosomes (2 M, 2 S) in side view of anaphase of *F. macrandra* ($n = 12$). $\times 1500$. FIG. 8—Polar view of anaphase in triploid *F. pudica*. Note the pair of daughter chromosomes, with a long constriction. The invisible centromere was presumably equidistant from the two arms, since they lay at the same level. $\times 2200$.

Evidently, therefore, the balance of the pollen grain is an important factor in its development in these plants, and the unbalanced types are largely eliminated.

3—ABNORMAL MITOSIS

The pollen grain mitosis in *F. pudica* may follow a normal course, and regular anaphases are found (fig. 8). But a proportion of the grains show to a varying extent and in various respects an abnormal behaviour at metaphase (fig. 9). In normal mitoses, when the chromosomes are

contracted to a characteristic length, they arrange themselves with their centromeres evenly distributed on the equatorial plate, and the centromeres divide at the same time, moving apart along the spindle arcs. In these abnormal mitoses the chromosomes fail to synchronize in the division of their centromeres, and they are *either* excessively contracted and normally distributed (A) *or* normally contracted and confined to the edge of the plate (B and C), to give the appearance of a "central spindle", such as is characteristic of mitosis in many animals.

There seems to be no connexion between variation in the number of chromosomes present and variation in their behaviour, which must

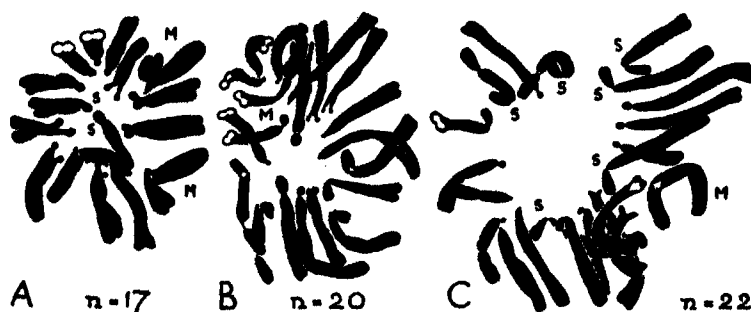


FIG. 9.—Three pollen mitoses of triploid *F. pudica* ($n = 17, 20$, and 22) graded according to the degree of spindle pole repulsion. Note the different stages of separation found in each nucleus. $\times 1800$.

therefore be considered the expression of a parental and not of a gametic character.

The lack of synchronization I have found also in *F. macrandra* associated with abortive and irregular anaphases. The central spindle is associated with the failure of some chromosomes to lie on the spindle at all (the precocious chromosomes in fig. 9, C). Their centromeres then separate, but not in an axial direction. This abnormality is of special interest, for it shows that in these cases orientation depends on the spindle while division of the centromere does not. The separation of the chromatids is therefore autonomous, as Belar said (1933): and the reason for this is that it is controlled by the centromeres, which are autonomous, and not by the spindle or centrosomes. Single univalents will similarly go through the motions of a second division in nuclei on their own, and therefore without spindles or centrosomes, in triploid *Hyacinthus* (Darlington, 1929, *b*). Acentric chromosomes—lacking centromeres as a result of crossing-over in inversions—will, on the other hand, show no movement at anaphase. Whether they are single chromatids at first anaphase (Richardson, 1936), or chromosomes derived from such chromatids in

the pollen grain mitosis, as I have found one in *Podophyllum*, they lag on the equator. The separation of the chromatids at early anaphase therefore depends directly on the repellent action of the centromeres, and this action is independent of the spindle, merely depending on it for its orientation and its efficiency.

How do these abnormalities of orientation, contraction, and division come to be related? The lack of synchronization between centromeres might depend on their spatial relationship with the spindle, but the variation in contraction can most easily be explained as a difference in the time during which the contraction can take place. C is then an earlier stage than A in regard to contraction (fig. 9). Now I have suggested that during anaphase the repulsion of the poles wanes. It might well follow that the polar repulsion was greater at the earlier stage, and that the chromosomes would be repelled to the periphery of the spindle, as is seen in C. I am therefore inclined to regard the abnormalities as due to the chromosomes being precocious in their development, in relation to the centrosomes or spindle, although not so precocious as in polymitotic maize (Beadle, 1932). This disadjustment of the time-relationship of chromosomes and spindle or centrosomes is then met by metaphase being either too late in relation to the chromosomes (A) or too early in relation to the spindle (C) or half-way between the two (B).

In this way these mitoses would be changed in the opposite direction to the anomalous meiosis to be described in *F. meleagris*, where I have assumed that the spindle is precocious relative to the chromosomes.

B—ABNORMAL MEIOSIS

There are four different sources of variation in the shapes and sizes of chromosomes seen in fixed preparations. All of these have their effect in *Fritillaria*. First there are differences due to fixation itself. Aceto-carmine fixation usually leaves the chromosomes unchanged in size. Fixatives of the Flemming type reduce their volume to about one-third, but this effect may vary according to the treatment. When a preparation is re-stained or re-mounted the size of the chromosomes is reduced still further (fig. 1).

Allowing for this source of variation, it seems that there is a characteristic variation between species and between varieties of the same species. This variation must be due to a genotypically controlled difference between the varieties. For example, two clones of *F. ruthenica* from different sources were found to differ in the size of the chromosomes, the

smaller type being smaller than any others in *Fritillaria* (Part II, figs. 10 and 11).

A third kind of variation is that found within a single preparation made from one flower. This may be due to one of the two remaining sources: differential effect of an environment within an anther, or genotypic difference between cells resulting from mutation. The first possibility must be borne in mind in considering this kind of variation, but I have no evidence that it is indeed effective. The second possibility becomes a certainty when the other has been excluded. This seemed to be the case in regard to a variation affecting the size of the chromosomes in *Tradescantia* (Darlington, 1929, *a*). It seems also to be the case in two cells in which I will now describe the first metaphase chromosomes.

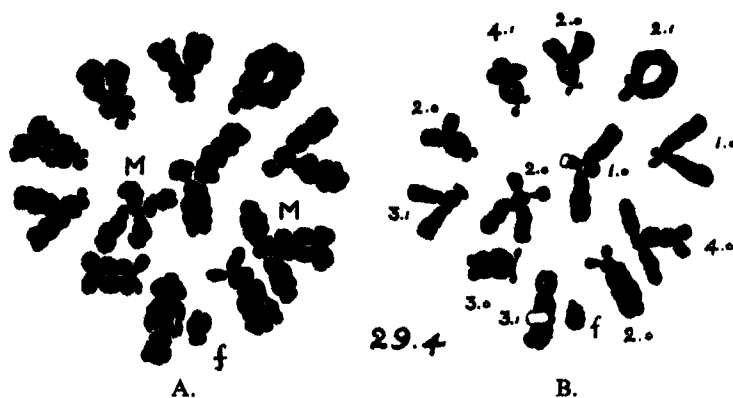


FIG. 10—First metaphase in a mutant pollen mother-cell of *F. pluriflora* with contracted chromosomes, before and after re-staining (*cf.* Plate 11). $\times 1600$.

They are surrounded by normal cells and their distinct properties must therefore be due to gene mutation following the previous mitosis.

The first cell is in *Fritillaria pluriflora* (fig. 10, figs. 18 and 19, Plate 11). This species, with semi-localized chiasmata, typically has long chromosomes like those illustrated in *F. acmopetala*, closely packed on the metaphase plate with some overlapping when seen in polar view. In the exceptional cell the chromosomes are more condensed than usual and widely separated on the plate, more so than in any other pollen mother-cell of *Fritillaria* that I have seen. It seems that there is a correlation between the formation of the spindle and the degree of condensation (spiralization) of the chromosomes, a correlation most simply explained by an upset of the time relationship of the two processes.

The second cell is in *F. Meleagris* (fig. 11, and Plate 13). It shows a more emphatic departure from the typical behaviour of meiotic chromo-

somes. This cell, like the other, has complete chromosome pairing with a normal number of chiasmata, normally distributed, but it is abnormal in respect of every other property distinguishing meiosis from mitosis, as follows:

(i) The chromosomes are nearly as long as at mitosis and nucleolar secondary constrictions are still visible. The constriction at mitosis is therefore retained as a result of the lesser contraction and is probably obliterated at meiosis only by the assumption of the major spiral, possibly by the joint coiling of the chromatids in the major spiral.

(ii) The chromatids are loosely associated so that chiasmata are less clearly identifiable, and the distinction between partner and non-partner

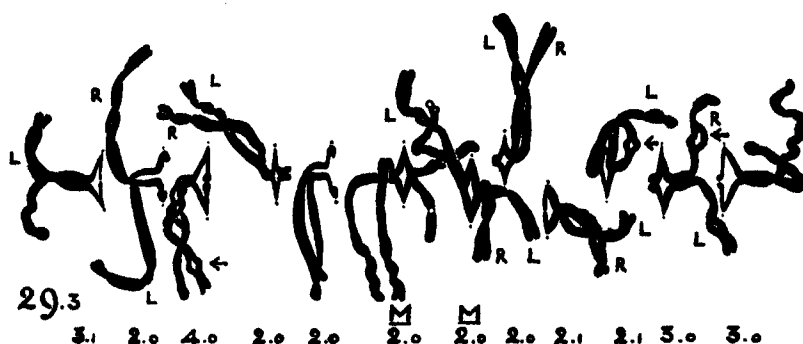


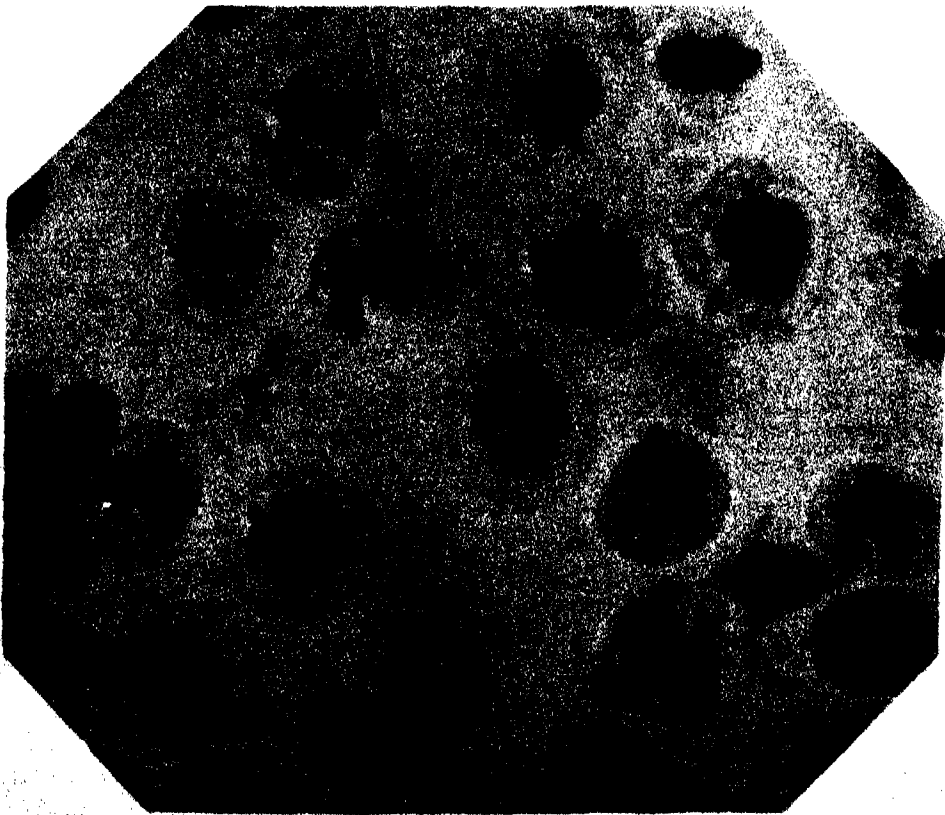
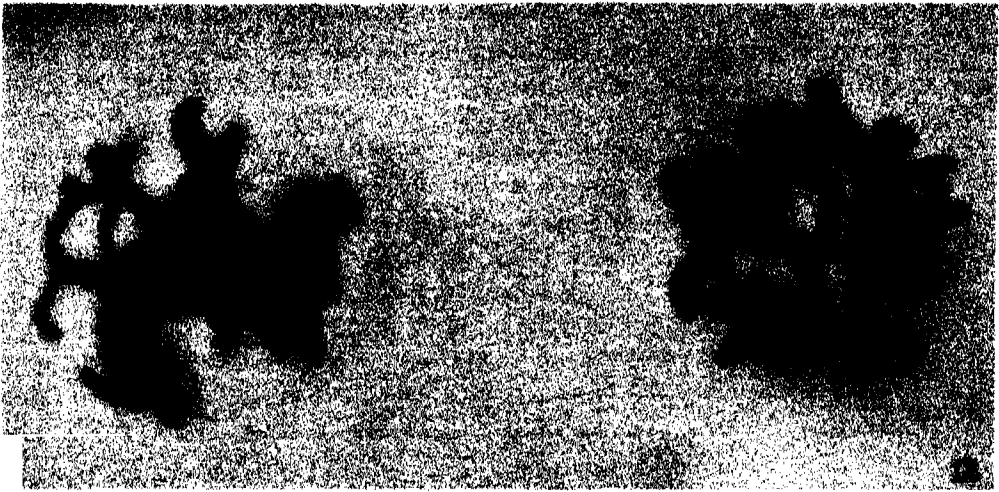
FIG. 11—Side view of abnormal first metaphase of meiosis in *F. meleagris* (cf. Plate 13). Numbers of total and terminal chiasmata given under each bivalent. Centromeres marked conventionally. Anomalous loops marked with arrows. $\times 1600$.

chromatids in small loops is reduced. At three points apart from chiasmata the paired chromatids were opened into small anomalous loops as though repelling one another.

(iii) The free arms of some of the chromosomes show relic coiling such as is seen at metaphase in ordinary mitoses and at diplotene, but not later, in meiosis (Darlington, 1935, *a*).

(iv) The chromatids are relationally coiled in many of the free arms, and owing to their loose association the direction of coiling can be clearly seen. It is consistent in particular arms. In six bivalents the coiling in both of the homologous free arms was clear and in every pair they coiled in opposite directions.

Let us consider first the coiling properties of the chromosomes. In organisms with freely distributed chiasmata very little chromatid coiling remains after chiasma formation (cf. Darlington and Dark, 1932). In *Fritillaria* with localized chiasmata I have assumed (1935, *a*) that a coiling



Typical and atypical meiosis in *F. Meleagris*. Fig. 12 $\times$ 2000; fig. 13 $\times$ 600.

of chromatids will persist at diplotene equal and opposite to the amount of coiling of chromosomes that persists, but owing to the close association of the chromatids this coiling cannot be seen. At metaphase it is still difficult to see whether coiling occurs. In intercalary segments which have had coiling of chromosomes at diplotene it should be increased by their uncoiling, in the free distal arms it is certainly absent or nearly so.

The opposite coiling of homologous arms at meiosis is inconsistent with the view that the coiling properties of chromatids are genetic characteristics of individual chromosomes. Yet its regularity in the six observed bivalents suggests that it is no accidental phenomenon. It indicates that the development of the relational coiling of chromatids may depend on other conditions which conflict in effect with the principal conditions that I have suggested (1935, *a*) but which I cannot yet identify.

The first three correlated abnormalities agree in suggesting a common cause, viz., that the nucleus has been interrupted at the diplotene stage by a precocious metaphase, *i.e.*, by a precocious development of the spindle. The spindle is known in many other cases to be controlled by conditions that are separable from those controlling the chromosomes, both genetically (Moffett, 1932; Beadle, 1933, *a*) and developmentally (Fankhauser, 1934).

The explanation of such special aberrations of mitosis and meiosis as I have described here cannot rest on the broad basis used for the induction of the timing relationship of meiosis and mitosis, and it cannot therefore have a comparable validity. A timing irregularity is, however, the simplest assumption to make.

SUMMARY

1—Counts show the relative inviability of the unbalanced pollen grains in triploid forms of *Fritillaria pudica* and *F. lanceolata* (unlike those of *Hyacinthus* and *Tradescantia*). Supernumerary fragments seem to be inert in this respect.

2—Two kinds of abnormal division show the potential independence of the intra-nuclear and extra-nuclear agents of divisions:

A—Triploid *Fritillaria pudica* has an association of abnormalities of mitosis in the pollen grains which is attributed to an upset of timing between the poles or centrosomes and centromeres. Precocious chromosomes may have centromeres dividing before they get on to the spindle and therefore unoriented. At the same time the strong polar repulsion produces a central spindle with the chromosomes confined to the edge.

B—At the first metaphase in two pollen mother-cells of smears of *F. pluriflora* and *F. Meleagris* the chromosome forms and arrangement was atypical, the one being less like mitosis, the other more so. These irregularities can be most easily understood as due to an upset of the time relations of the centrosome or spindle and chromosome cycles.

The External Mechanics of the Chromosomes

V—THE BALANCE THEORY OF MITOSIS

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(Communicated by Sir Daniel Hall, F.R.S.—Received 15 April, 1936)

In the present studies, material of *Fritillaria* has been used to show the effect of different variables on the external movements of the chromosomes. The first shows the effect of different time-relationships at meiosis on the localization of pairing. The second shows the effect of the body-repulsions on the metaphase arrangement of bivalent chromosomes. The third shows the effect of the centromere-repulsions on the metaphase arrangement of trivalents and their relationship with the polar repulsions and the axial orientation of the spindle particles. The fourth shows the effect of varying the polar repulsions, by varying the time co-ordination of centrosomes (or spindle-pole determinants) and chromosomes, on the metaphase arrangement at mitosis and meiosis.

These and more general observations in other material are the main sources of evidence that I want to consider in relation to the external mechanics of the chromosomes. It is impossible to describe here the evidence of every kind of force working on every kind of chromosome configuration at every stage of development. To do so would make the material unwieldy. We should not be able to see the wood for the trees. Nor is it yet possible to deal thoroughly with those special types of mitosis found in the Protista or those exceptional types of meiosis described by the Schraders (1931, 1932) and by Metz, Moses, and Hoppe (1926), since the general rule must be understood before the exceptions can be explained. I therefore propose to consider merely that great body of plants and animals which seems to obey certain consistent rules at meiosis and mitosis.

The first conclusion to be drawn is that the metaphase arrangements and anaphase movements reveal the action of the same forces as are shown by the prophase arrangements and movements. We see that, at all stages, two sources of repulsion are working, one localized at the centromere, the other diffused over the whole body of the chromosome. This conclusion is in harmony with the main theme of the precocity theory of meiosis, viz., that the forces working on the chromosomes at all stages of meiosis and mitosis are of the same kinds—specific attractions and non-specific repulsions.

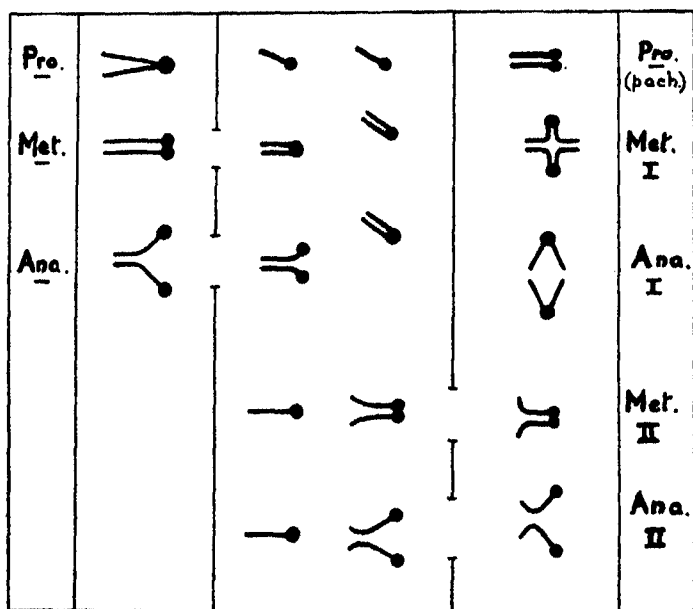


FIG. 1—Diagram showing the division cycle of the centromere in relation to that of the chromosomes at mitosis (left) and for bivalents (right) and univalents (middle) at meiosis.

The second conclusion to be drawn carries the conception of the relatedness of forces much further. The positions of the two centromeres of a bivalent at metaphase are like those of two daughter chromosomes at anaphase of mitosis. But while the mitotic position is rapidly changing the meiotic position is temporarily stable. It is a position of equilibrium between the two centres of repulsion at the spindle poles, the centromeres, and the two centres of repulsion on the bivalent chromosomes, the centromeres. The positions of equilibrium are modified, first, by the tension of the parts of the chromosomes connecting the centromeres of each configuration, which is greater the shorter the distance between the

centromeres, and secondly, by the effects of the body repulsions. These can both be identified by the asymmetry they produce in bivalents and also in trivalents (figs. 1 and 2).

These observations suggest that the centrosomes and centromeres are acting in the same way. When we find that the centromeres can modify the structure of the spindle which has been determined by the centrosomes, both at metaphase and anaphase, and that a centromere can determine the direction of the cell-plate formed between two bodies of chromosomes, we can be in no further doubt of the relatedness of the two kinds of bodies.

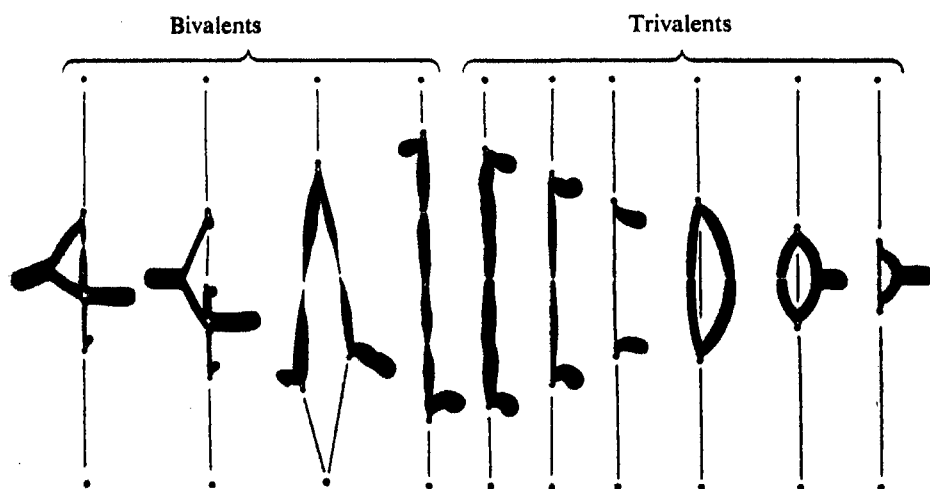


FIG. 2.—Diagram showing the equilibrium positions of the centromeres in relation to the centrosomes in bivalents and trivalents at meiosis with different positions of chiasmata. These positions are modified by the tension of chromosomes and by their body repulsions. The body repulsions of other chromosomes are neglected.

On this view Belar's observations of spindle behaviour become intelligible (1929, *a* and *b*). At anaphase, the spindle stretches; the middle part, Belar's "Stemmkörper", has a different shape after the movement of the chromosomes because it is no longer a centrosome spindle, expanded in the middle by a metaphase plate; it is simply a *centromere spindle* formed between two circular plates, instead of between two points, and therefore cylindrical instead of spindle-shaped (fig. 3).

In some protozoans such as *Aulacantha* (Belar, 1926, fig. 56) and in the micronuclei formed at meiosis by lagging chromosomes (Darlington, 1929, *a*, fig. 41), division takes place without any centrosomes or localized poles and the spindle is purely a centromere spindle, cylindrical from

the beginning. Similar observations of spindles formed independently of any polar centre in the Coccidae have led the Schraders (1931, 1932) to a similar conclusion, viz., that these spindles are determined by the chromosomes and, in fact, by each chromosome separately. The relationship of these observations to the present studies are shown by the work of Gross (1935) on *Artemia salina*. In the first meiotic division of the egg of this animal, a purely intra-nuclear spindle is formed, i.e., a centromere spindle. In the early cleavage divisions it seems that spindles are separately formed within and without the nucleus, to combine later when the nuclear-cytoplasmic surface breaks down. Here we see at once the independence, the mechanical similarity, and the mutual replaceability of the spindle-forming functions of the centrosomes and centromeres.

At metaphase of meiosis or mitosis the centromeres of the chromosomes lie half-way between the poles in a flat plate, giving equilibrium between the repulsions of the two centrosomes, or in a spherical plate where only one centrosome is present, the opposite repulsion arising presumably from the cell-wall (Belar, 1933). At anaphase, on the other hand, the centrosome repulsions merely retard the chromosomes, and as they move apart the repulsions evidently wane rapidly. Anaphase movement therefore depends on a relation between the centromeres of the separating chromosomes and not on the activity of the centrosomes. This is shown in anaphase where the metaphase plate has lain near to the cell-wall, as I have found it may in pollen-grains (e.g., *Podophyllum versipelle*, Darlington, 1936, a). One group of chromosomes then reaches the limit of movement, close to the wall in the middle of anaphase; the other group continues to move away from the first—presumably owing to the stretching of the centromere spindle, as Belar suggested (1933). Thus, in spite of their similar activities, centrosomes and centromeres have widely different effects.

When we look for other evidence of the likeness of centrosomes and centromeres we find that each goes through a division cycle correlated with that of the chromosomes; but the cycle of activity of the centrosome being relatively earlier in meiosis than in mitosis, it divides twice for the chromosomes' and for the centromeres' once. Each has a maximum repulsion after division; each is variable in its staining properties at different stages and in different organisms, its existence being hypothetical in many organisms possibly owing to its small size. Why, then, it may be asked, do they seem to have such an entirely different place in the mechanism of cell-division? The answer seems to be that one is attached to the chromosomes, the other is not. In the higher organisms the centrosome

has left the nucleus and has acquired a control over the external processes of cell-division which it has not attained in some of the simplest organisms (*cf.* Belar, 1926). It lives in a different substrate, the cytoplasm, and has therefore a different cycle of division and activity and a different effect on this substrate. It can produce an orientation of molecules in the

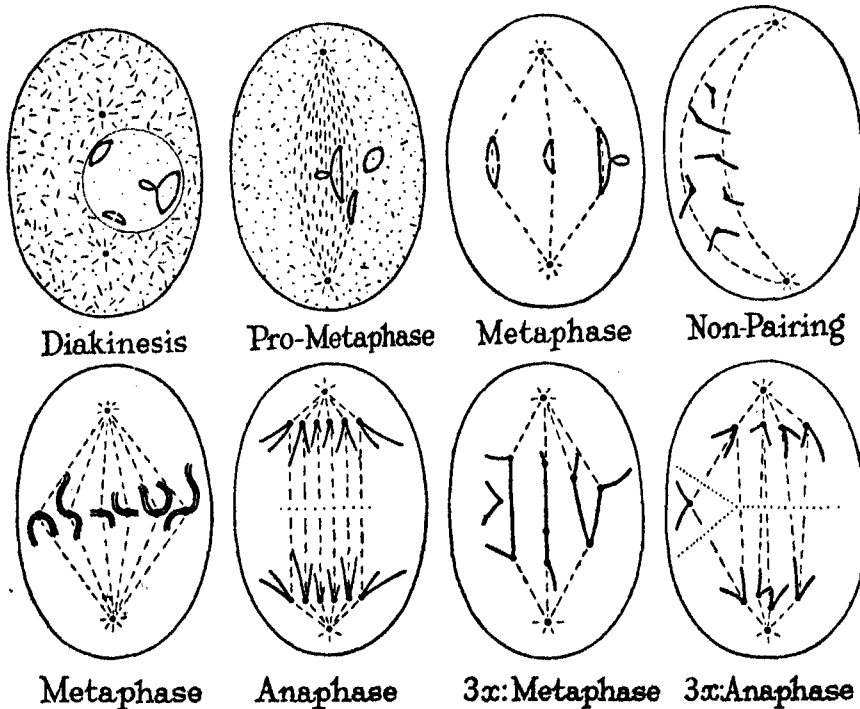


FIG. 3.—Diagram showing (i) the action of the centrosomes in orientating long molecules to make a spindle, (ii) the spindle's action on the bivalent chromosomes at pro-metaphase, (iii) the action of the bivalents in broadening the spindle at metaphase, (iv) the action of the spindle in lengthening itself in the absence of broadening by the metaphase plate owing to non-pairing, (v) the mitotic metaphase, (vi) the change in shape of the spindle at mitotic anaphase, (vii) the orientation of trivalents on the spindle, and (viii) the disjunction of trivalents and the formation of a plate under the action of the centromere-spindle.

cytoplasm such as the centromeres, when they are shut up in a semi-permeable nucleus, cannot produce.

The particular difference between cytoplasm and nuclear sap that is concerned is made clear by the conditions of formation of an intranuclear spindle at meiosis in *Artemia* (Gross, 1935). The nucleus contracts before the spindle forms, presumably by loss of water. There is therefore, no doubt, a threshold concentration of the specific molecules for

spindle-formation, a concentration which is not reached within the nucleus in the higher plants.

The cytoplasm must also differ from the nucleus in having a different p_H cycle. That of the cytoplasm, we must suppose, governs the related surface-charge and division cycles of the centrosomes; that of the nucleus the corresponding cycles of the centromeres and chromomeres, and hence the changes in molecular coiling or the spiralization cycle of the chromosomes (figs. 3 and 4; cf. Darlington, 1935, *a*).

The cycle of division and activity of the centrosomes anticipates that of the centromeres, whose movements are therefore at first subordinate.

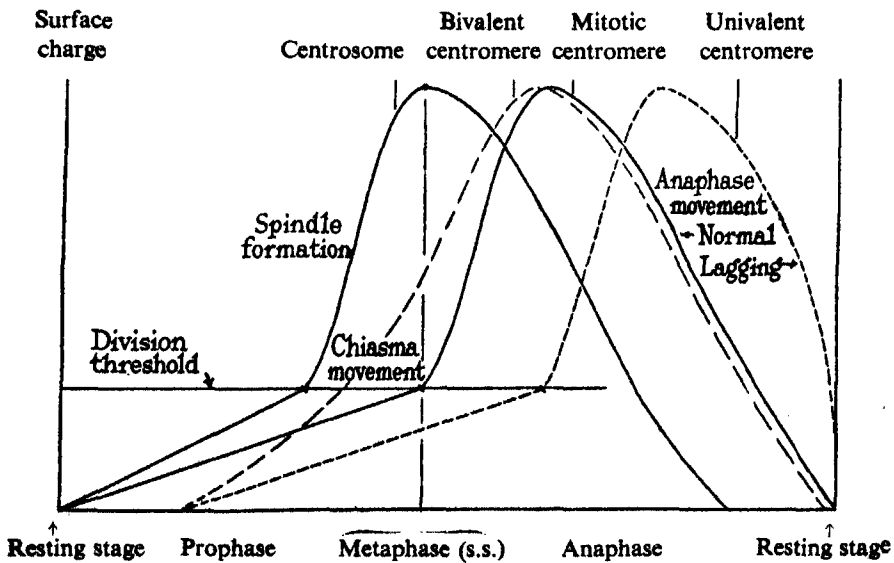


FIG. 4—Graph to show the time-relation of the centrosome and centromere cycles of surface-charge with one another, with chromosome movements, and with their own division, at mitosis and at meiosis with bivalents and univalents.

The centrosomes orientate the spindle molecules, the spindle molecules determine the orientation of the centromeres, which in turn modify the shape and orientation of the spindle. All this necessarily depends on accurate time-coordination. The last studies show the kind of errors that result from upsetting this coordination. They show that the timing change represented by the difference between meiosis and mitosis is only one of the changes possible between the physiologically separable units in the system—centromeres, centrosomes, and chromosomes.

These hypotheses make it possible to resolve the two great contradictions of mitosis which are, first, that the chromosomes move away from

the poles of the spindle on to the metaphase plate but never move off the spindle, and secondly, that the chromosomes, which move away from the poles before metaphase, move towards the poles after metaphase. Together the hypotheses make up a theory of chromosome movement on the spindle in meiosis as well as mitosis, depending on the balance or coordination of related centres of repulsion.

This theory, which may be described as a *Balance Theory of Mitosis*, depends on five new hypotheses apart from those implied by the precocity theory of meiosis:—

(i) Every chromosome includes in its linear structure a body, the *centromere*, which divides at the metaphase following the resting stage when the chromosomes divide. This body passes through a cycle of varying surface charges determining repulsion which reaches a maximum at anaphase.

(ii) Some nuclei have a body, the *centrosome*, associated with them. This body has a similar cycle of activity to the centromere, but it is earlier, dividing before metaphase and reaching its maximum repulsion at metaphase. The division and repulsion cycles of centrosome and centromere are correlated.

(iii) The cytoplasm, but not the nucleus, contains particles (probably chain molecules with associated water) whose dielectric properties make them capable of orientation in an electro-magnetic field in such a way as to transmit the acting repulsions most effectively.

(iv) This orientation constitutes the spindle, whose dielectric properties compel the paired or double centromeres of the chromosomes to orientate themselves at metaphase on the spindle, and in the same axes as the molecules of the spindle, in spite of the repulsion of the centrosomes.

(v) The typical course of mitosis and meiosis therefore depends on a *balance*: the division and repulsion cycles of the centrosome anticipate those of the centromere by a fraction of a cycle in mitosis and by a whole cycle in meiosis, where the precocity of the centrosome is correlated with that of the other external factors in division.

This theory, like the precocity theory, rests on the assumption of an independence of conditions inside and outside the nucleus which is to be inferred as much on the general ground of the semi-permeable properties of the nucleo-cytoplasmic surface as from the particular morphological evidence I have adduced. The precocity theory assumes a unity of the mechanical properties of meiosis and mitosis. The balance theory assumes, in addition a unity, of the mechanical properties of the two kinds of repulsion centre, the centrosomes and the centromeres. Variations in the type of mitosis are then due to the replacement of the centrosomes by undefined agents which are analogous in effect.

GENERAL SUMMARY

Among 24 species of *Fritillaria*, 15 have localized chiasmata, 5 have triploid as well as diploid forms, and 5 have supernumerary fragments. The distribution of the localized chiasmata shows the mechanics of pairing at zygotene and especially that the two ends of chromosomes often pair before the middles. The asymmetrical arrangement of bivalent chromosomes on the metaphase plate shows the effect of the repulsions of other bivalents. The arrangements of trivalent chromosomes show how the centromere or dynamic centre of the chromosome modifies the simple orientation of the spindle, and also how the centromeres reach an equilibrium position between their own repulsions and those of the spindle poles or centrosomes. Abnormal behaviour at mitosis in the pollen grains, and at meiosis, shows a correlation between the contraction of the chromosomes, the repulsion of the centrosomes, and the division and orientation of the centromeres. Such a correlation suggests an upset in the timing relationships of these processes.

Taken together with experimental work on the centrosomes and spindle, and studies of chiasma movement during prophase, these observations lead to the conclusion that chromosome movement outside the nucleus is controlled by the activities of the centrosomes and centromeres. These activities consist in coordinated cycles of division and repulsion; the centrosomes first determine the spindle, and reach their maximum at metaphase, the centromeres modify the spindle at metaphase and, reaching their maximum at anaphase, convert it into a centromere spindle, of different shape.

These hypotheses are an attempt to resolve the contradictions between the movements of metaphase and anaphase, as well as of meiosis and mitosis, by describing them all in terms of a consistent physico-chemical system.

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The Structure of Nerve Fibres in Cephalopods and Crustacea

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(Communicated by Sir Henry Dale, F.R.S.—Received 20 May, 1936)

[PLATES 14, 15]

INTRODUCTION AND METHODS

The nerves of Crustacea have been used for many years for physiological and biochemical investigations, but the value of the results obtained has been seriously reduced by the absence of reliable data as to their histological structure. The present paper attempts to describe the axons and their sheaths not only in a Crustacean, *Maia*, but also in Cephalopods, which may prove to be very suitable material for the investigation of the properties of non-medullated nerve, and in which the giant nerve fibres provide exceptional opportunities for study of the axon.

Most of the observations were made with *Maia* and *Sepia*, which were obtained from the laboratories of the Marine Biological Association at Plymouth, and my thanks are due to Dr. E. J. Allen and his staff for their assistance. The histological work was done in the Department of Zoology and Comparative Anatomy at Oxford, and I am most grateful to Professor E. S. Goodrich for his advice during the course of the work.

I have also to thank Professor A. V. Hill for suggestions as to the types of histological data required.

The nerves of *Sepia* which are most convenient for study are those running to the mantle, shown diagrammatically in fig. 1. To prepare them the animal is killed by decapitation and laid on its back. The mantle is opened by a midventral cut and the fin nerve on each side is then seen running past the stellate ganglion, whence it can easily be dissected through the muscles on to the back. In this way a stretch of nerve more than 10 cm. in length can be obtained from a large *Sepia*.

In fig. 1 are shown on the right the connexions of the giant fibres, on the left those of the smaller neurons. The main nerve leaving the C.N.S. for the mantle is the mantle connective or pallial nerve (*m.c.*). This gives off first a medial branch to the retractor muscle of the head (*n. retr. cap.*), then the large fin nerve (*fin. n.*); finally it runs into the stellate ganglion (*st. gn.*), from which stellar nerves (*st. n.*) radiate to the mantle muscles (*mant. musc.*). Afferent fibres (*aff.*), having their cell bodies at the periphery, run both in the fin nerve and stellar nerves, from which they pass through into the pallial nerve, probably without synapse in the ganglion. The efferent path of all fibres to the mantle muscles contains a synapse in the stellate ganglion, but the fibres to the chromatophores (*cr.*) run through the ganglion without synapse. For further details see Sereni and Young (1932) and the literature there quoted.

For histological study all nerves were removed from the animals a few minutes after death, and were fixed stretched at approximately their natural length on pieces of card or tied to capillary tubing. The methods used for fixation and staining are described in the text. The best results were obtained by dissolving the fixing substances in sea water (see Young, 1935, *a*).

THE SHEATHS OF THE NERVE FIBRES OF CEPHALOPODS

The sheaths surrounding the axons in the peripheral nerves of Cephalopods have already been described by Sereni and Young (1932) as composed of continuous sheets, containing nuclei, and apparently consisting of connective tissue. In that paper the axons were described as being sometimes much smaller than the connective tissue tubes in which they run. This appearance is now known to be an artefact. In a properly fixed Cephalopod nerve each axon, large or small, entirely fills its sheath, and that this is the normal condition in all states of extension of the nerve can be verified by observation of unfixed fibres examined in sea water. Under such conditions, no free space can be seen between the outer surface of the axon and the inner surface of the sheath. Nuclei of the

sheath, lying in its very innermost layers, may indent the surface of the axon, and at such points it can be seen most clearly, both in living nerves

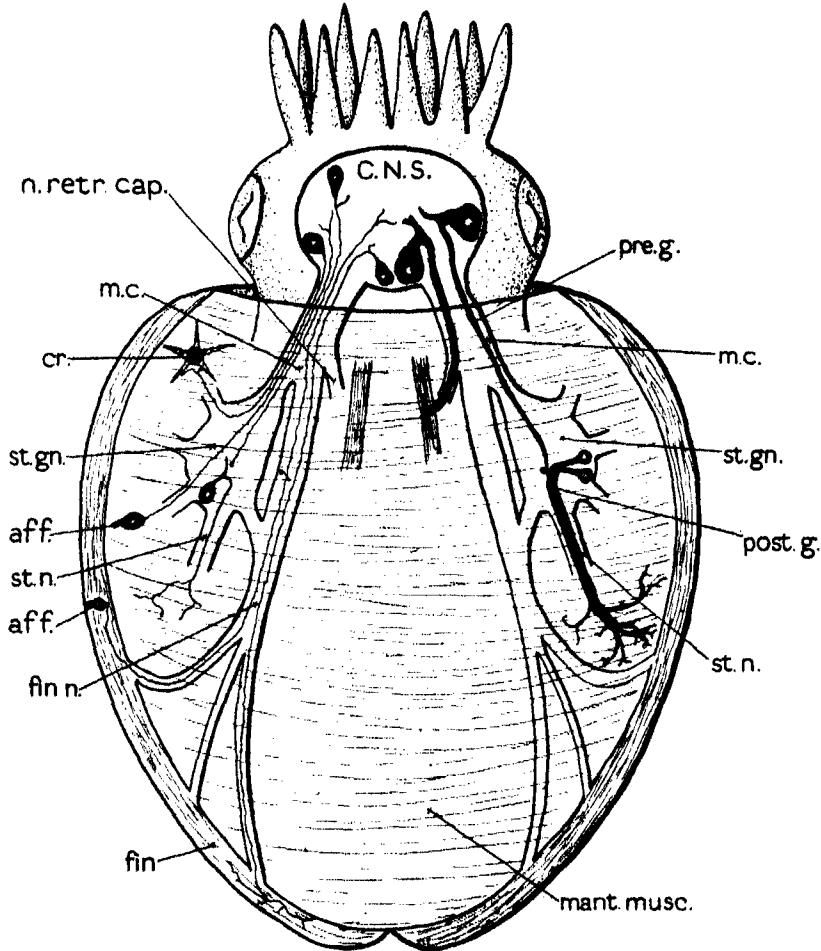


FIG. 1—Diagram of the innervation of the mantle in *Sepia officinalis*. The details of the connexions of the giant fibres are not known; *pre. g.*, represents the "preganglionic" fibres, which arise in the palliovisceral ganglion; *post. g.*, the "postganglionic" fibres, which take origin from the fusion of the processes of several cells. For further explanation see text.

and suitably fixed preparations, that there is no trace of any intervening fatty sheath (figs. 5, 6, 7, Plate 14).^{*} Indeed, these innermost sheath nuclei

^{*} Since the MS. of this paper was written, it has been shown by the use of polarized light that a thin layer of oriented fatty molecules is present *outside* the layer whose nuclei indent the axon (Bear, Schmitt, and Young, *unpublished*).

sometimes lie so close to the axon as to appear to be part of the latter. Careful observation will show, however, that they are surrounded by a very thin layer continuous with the rest of the sheath. Examination of teased nerves, and of longitudinal sections shows that the sheath is continuous over the whole surface of the fibre and that there are no gaps comparable with the nodes of Ranvier (fig. 9, Plate 14).

After fixation in OsO_4 or fluids which contain it, the whole sheath may appear brown or grey, but there is no trace of the homogeneous black ring so characteristic of vertebrate medullated fibres. That the sheath consists of connective tissue is suggested by the fact that it is formed of a number of fibrous sheets staining with anilin blue and with van Gieson's stain and by the appearance of the nuclei which it contains. However, such criteria are inadequate to allow certain identification of the tissue, and it is possible that the inner nuclei, lying close to the axon, are of different origin from the rest. No clear distinction between separate layers has been seen, and it seems probable that the whole thickness of the sheath consists of collagenous sheets. Further embryological and chemical investigations are needed, however, before we can be certain whether the whole sheath is mesodermal, or whether anything corresponding to the ectodermal Schwann sheath is present.

In order to obtain some estimate of the proportion of the nerves which is occupied by the axons, stellar nerves, fixed in formaldehyde, sectioned transversely at $3\ \mu$ and stained with the azan method, were photographed at magnifications of 90 and 660 diameters (fig. 8, Plate 14). The outline of a whole nerve was then carefully cut out on a print and the piece weighed. First the perineurium and secondly the giant fibres were then cut out and the remainder weighed after each operation, thus providing estimates of the proportion of the cross-sectional area which is occupied by these structures. The area occupied by the small fibres was estimated by cutting out and weighing the axons contained in a portion of the photograph magnified to 660 diameters.

By this method the perineurium was found to comprise 8% of the cross-sectional area of a larger stellar nerve and 12% of that of a smaller one, while the giant axons made up 13% of the former and 12% of the latter nerve. The smaller axons occupied 74% of the area examined, which was chosen so as not to include any perineurium or giant fibres.

No doubt the density with which the axons are packed varies in different areas of any one nerve and between different nerves, but since the fin nerve is very similar to the small-fibre portion of the stellar nerves it is probable that about 65% of the cross-sectional area of the fin nerve is occupied by axoplasm, 10% by the perineurium, and the remaining 25% by other

tissues, including the sheaths of the axons, the grosser bundles of connective tissue, the blood vessels and spaces containing tissue lymph. In the stellar nerves, on account of the presence of the giant fibres, the proportion of axoplasm is somewhat greater.

SIZES AND FREQUENCIES OF FIBRES

The axons of *Sepia officinalis* vary from minute fibres of less than $1\ \mu$ diameter to giant axons as much as $200\ \mu$ across. In *Loligo* the giant axons in the stellar nerve may be nearly 1 mm. in diameter.

Estimates of the numbers of fibres of the various sizes in the nerves were obtained by photographing transverse sections of nerves, fixed in formaldehyde and stained by the azan technique, at a magnification of 200 diameters, and then measuring and counting the fibres on the prints. The diameters measured were those of the connective tissue tubes, not of the axons, since these latter are often much shrunken. The number of fibres in the parts of the pallial nerve is so great that a sampling method was adopted, four rectangles being taken at right angles, each along a radius of the nerve from near the centre to the perineurium. The fibres in each area were measured, counted, and grouped by intervals of $5\ \mu$, and the four frequency distributions thus found compared by means of a χ^2 test (Fisher, 1930). The test showed that the fin nerve is sufficiently homogeneous for this method of sampling to be appropriate, but for the nerve to the stellate ganglion the differences between the different rectangles are greater than would be expected from errors of random sampling. The difference, however, lies mainly in the proportion of the fibres less than $5\ \mu$, and is not likely to affect any conclusions which can be drawn at present from the shape of the size-frequency distribution.

Estimates of the total numbers of fibres in the nerves were then made by discovering the proportion which the areas counted bear to the whole areas of the nerves, and the standard deviations of the means of the numbers in the four samples of each nerve calculated, in order to give some measure of the accuracy of the estimates. Two fin nerves were treated in this way and the numbers of fibres agreed within the limits of accuracy of the method.

In the nerve to the stellate ganglion there is a bundle of giant axons segregated by themselves in a corner of the nerve. Since this bundle contains only very few smaller fibres it was treated separately from the rest of the nerve, its fibres being measured and counted individually. For comparison this giant fibre bundle was counted in two separate nerves.

TABLE I—COMPOSITION OF NERVES OF *Sepia officinalis*

Nerve	Total fibres	< 5 μ	5-10 μ	10-15 μ	15-20 μ	20-25 μ	25-30 μ	30-35 μ	35-40 μ	> 45 μ
N. pinnae (<i>Sepia</i> DI)	7409 $\pm$ 855	%	42.3	23.0	14.8	9.5	7.0	2.2	1.2	
n. pinnae (<i>Sepia</i> GI)	6080 $\pm$ 456	%	38.7	27.8	20.4	10.5	2.6			
n. to st. ganglion (excl. giant fibres)	8500 $\pm$ 556	No.	5645	1547	629	280	212	68	51	9
Giant fibres	48	No.					9	9	3	2
Whole nerve (<i>Sepia</i> DI) ..		%	66.0	18.1	7.4	3.3	2.6	0.9	0.6	0.4
Giant fibres (<i>Sepia</i> GI) ..	47	No.					11	9	6	14
Stellar nerve	338	No.	162	145	28	1			1	1
		%	47.9	42.9	8.3	0.3			0.3	0.3

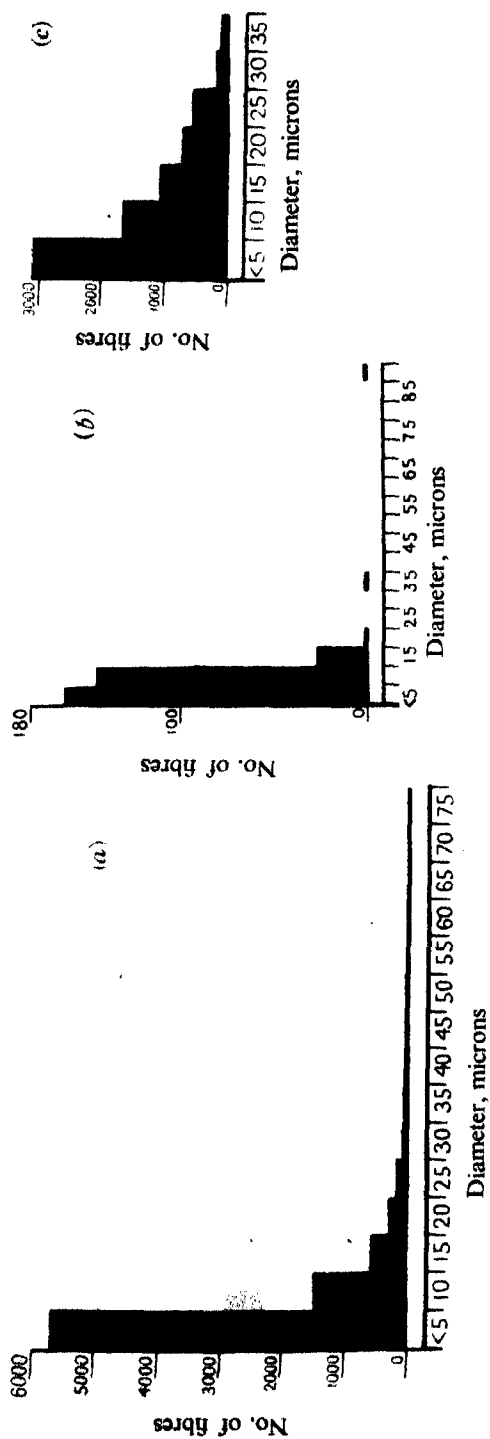


FIG. 2.—Sizes and frequencies of fibres in some nerves of *Sepia officinalis*. Measurements made on material fixed in formaldehyde and embedded in paraffin, in life all the fibres would be somewhat larger. (a) Nerve to stellate ganglion; (b) stellate nerve; (c) fin nerve.

Table I and fig. 2 summarize the results of these measurements. In all the nerves there is a continuous distribution of fibres from the smallest to 20 μ or 30 μ , proportionately more of the smaller fibres being present in the nerve to the stellate ganglion than in the other nerves. The smallest of the giant fibres in the nerve to the stellate ganglion are no larger than the largest fibres outside the giant fibre area, so that the distribution is continuous up to 75 μ . In the stellar nerves, on the other hand, there are fewer giant fibres, and these are much larger than any others in the nerve. The fin nerve contains no giant fibres.

THE AXONS OF CEPHALOPODS

The giant fibres of the stellar nerves provide especially favourable material for the study of the structure of the axons. Their connexions have already been briefly described (Young, 1936), and further details will be published shortly. It is important here to notice that they are not the axons of single cells, but are syncytia produced, in the case of the largest fibres of *Loligo*, by the fusion of the processes of many hundreds of small cells.

The structure of the sheath appears to be similar in all fibres, whether large or small. The sheath is absolutely thicker around the larger fibres, but does not appear to increase proportionately with the fibre diameter, so that the larger fibres have relatively the thinner sheaths.

In order to examine the structure of the axons, one of the stellar nerves is taken from a *Sepia* or *Loligo* which has just been killed by decapitation, and is teased out with fine needles in the animal's blood or a drop of sea water. One or more of the giant fibres can usually be separated out in this way and studied with the highest powers of the microscope. The axon then appears as a homogeneous cylinder, having no central core or definite neurofibrils which can be traced separately. There is, however, a very delicate longitudinal striation, extending throughout the thickness of the fibre. The striae are very fine and exceedingly numerous. Since, as will be shown below, coarser striations appear after damage to an axon, it is difficult to be certain whether this faint fibrillation be not also an artefact. Dr. B. Sen has recently examined giant fibres of *Sepia* with dark ground illumination, and he informs me that no fibrillation was to be seen under these conditions, the axon containing only a number of granules in Brownian movement.

When a giant fibre is severed, the substance of the axon may flow out from the cut end of the tube formed by the connective tissue sheath, fig. 10, Plate 14 (Young, 1935, *b*). As will be seen from fig. 3, the flow

begins soon after the cut has been made, and the axoplasm pours out into the surrounding medium while the sheath slowly collapses. While this outflow is going on, movements can be seen in the axoplasm at a distance of several millimetres from the cut surface. The flow is seldom uniform across the whole cross-section of the fibre, being sometimes faster at the edges, sometimes at the centre, depending apparently on the shape of the opening. When the flow is fastest at the edges, it sometimes results in the leaving of a central core of altered axoplasm, which only partly fills the connective tissue tube (*see* p. 329).

Very noticeable changes take place in the finer structure of the axon close to a cut surface. The fibrillation becomes much more distinct in the axon which remains inside the sheath, so that it is sometimes possible

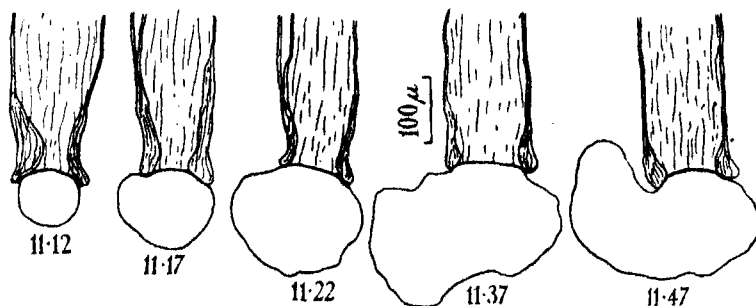


FIG. 3—Camera lucida drawings of the cut end of a giant fibre of *Sepia officinalis*, the cut having been made at 11·05 a.m. The outflow is often faster than in this case

to recognize distinct strands or fibres in such a region. As the stream emerges at the cut surface, this longitudinal fibrillation disappears. Coarser granules appear in the exudate, these being apparently artefacts, not present in the intact axon. The substance which emerges does not mix very readily with sea water, though it will do so if disturbed, but, on the other hand, it does not become separated from the water by the formation of any visible surface membrane. Brownian movement, which is not visible by transmitted light in the normal axon, appears in the exudate, especially at its outer edges.

All of these phenomena are seen equally well if the sheath of the axon is punctured by means of a needle at some point along its length. As shown in fig. 4, there is then a lateral outflow of axoplasm and the fibrillation becomes much more distinct in the affected region, with lines which follow the lines of flow.

These changes in the appearance of the axoplasm close to an injured point are a warning against placing too much reliance on observations of the visible or physical structure of axons which have been injured, or

punctured with microdissection needles. A fibre removed from the body to sea water maintains for some time a constant appearance, except, of course, close to its cut ends: but the operation of removal is necessarily a drastic one for the fibre, and we cannot even be sure that the appearance seen under these conditions is that of the fibre in the living body, though all the indications point to this being so.

Any injury to the sheath rapidly produces the fibrillation and granulation reported above, and the axoplasm has then certainly changed its properties. The fact that the axoplasm flows out from the cut end of an

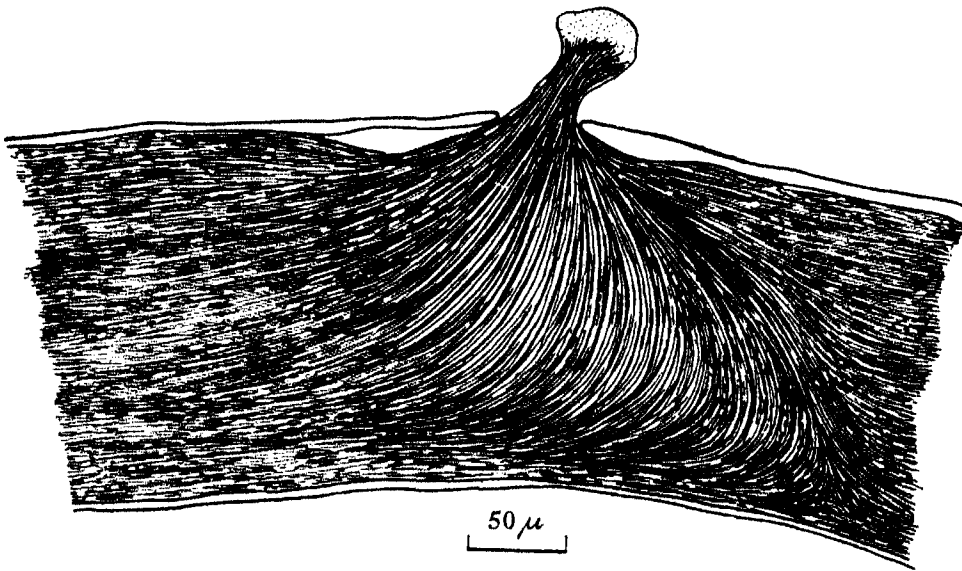


FIG. 4—Giant axon of *Sepia officinalis* which has been punctured laterally by a fine needle. The main lines of fibrillation were drawn with a camera lucida, but the details are diagrammatic. Note that the fibrils are more definite close to the injured point.

axon does not, therefore, make it certain that it is fluid in the living state, though this is suggested by the fact that the flowing movements can be observed in the axoplasm at some distance from the cut. These observations indicate, then, that the giant axons consist of a rather viscous fluid, whose substance shows some longitudinal organization but no definite continuous neurofibrils, though these latter readily appear under abnormal conditions.

Although these giant fibres are syncytia, originating by the fusion of the processes of many cells, yet no trace of their composite nature can be

detected either by study of the living, intact axons, or of those from which the axoplasm is flowing out, or in fixed preparations examined in longitudinal or transverse section. It is concluded, therefore, that the fusion of the separate axons is complete, the result being a giant axon, which functions as a single unit. Further details of this remarkable condition will be published shortly.

It is to be noticed that neither by observation of the intact giant fibres, nor by watching the process of outflow from them, does there appear any evidence of the existence of a surface membrane optically separable from the contents of the fibre. No doubt the molecules at the outside of the fibre take on special orientations to form a limiting membrane whose progressive depolarization probably constitutes the nerve impulse, but there is no reason to suppose that such a surface region of orientated molecules would be optically detectable.

AXONS IN FIXED PREPARATIONS

The study of fixed preparation of Cephalopod axons confirms the above interpretation based on the study of the living nerves. When well fixed by solutions having the appropriate saline constitution the axon completely fills its sheath, as in the living state, and the axoplasm appears to be homogeneous, except for a faint, wavy, longitudinal striation. No definite neurofibrils traceable for long distances are visible.

The faint longitudinal striation appeared after all the histological methods used, including fixation in Flemming's fluid, with or without acetic acid, formaldehyde, Bouin's fluid, mercuric chloride, with and without acetic acid, and various modifications of the methods of Cajal and Bielschowsky. The appearance varied somewhat according to the fixation, the striation being, for instance, somewhat coarser after fixation in formaldehyde (fig. 11, Plate 14) than after Flemming's fluid, which gave an appearance closely comparable with that of the living fibres.

None of the techniques employed produced in the axons any appearance of grosser neurofibrils traceable for long distances in the axon. In some of the preparations, however, there appeared central cores with a different staining reaction, and a more fibrillar appearance than the rest of the axon. Such cores were never seen in living intact nerves, and they appear to be artefacts, due to the currents and eddies which are set up in the axoplasm by the outflow from the cut end of the fibre. They occur close to the cut end of a nerve, and often take on remarkably complex forms (fig. 12, Plate 15). Sometimes these more darkly staining cores may stand out very distinctly, as, for instance, in the case shown in fig. 13, Plate 15,

where the core is markedly basophil and appears sharply demarcated from the rest of the axoplasm.

SMALLER AXONS OF CEPHALOPODS

The smaller nerve fibres in the mantle connective, fin nerve and stellar nerves, are essentially similar to the giant fibres. When examined in sea water they are seen to consist of an axon entirely filling its connective tissue sheath. The axon has no visible surface membrane and shows a faint longitudinal fibrillation. Comparable appearances are seen in fixed preparations (figs. 5, 6, 7, Plate 14).

When these smaller fibres are severed they do not immediately flow out from the cut ends of their sheaths, presumably because of the surface tension, which would tend to restrain the outflow of axoplasm from a narrow tube, while allowing it from a wider one. The initial stages of regeneration of these fibres have been studied by making preparations of the stumps a few hours after they had been cut, and it appears that the first stages of the process consist of an outflow of axoplasm, essentially similar to the faster outflow from a giant fibre.

SHEATHS OF NERVE FIBRES IN CRUSTACEA

In *Palaemon* and related genera, and in Mysids, the nerve fibres are surrounded by a fatty sheath which was first discovered by Retzius (1888 and 1890) and later investigated by Göthlin (1913) and Nageotte (1922). These sheaths are said to differ from those of Vertebrates in that (1) the sheath is only thinned and not completely interrupted at the nodes of Ranvier; (2) there are no incisures of Schmidt-Lantermann; and (3) the "myelin" is separated from the axon by a nucleated inner sheath.

The nerves in the walking legs of Lobsters and Crabs, however, are commonly supposed to be "non-medullated" (see Mangold, 1905, and Retzius, 1890, for early literature), but Lullies (1933) claims to have demonstrated myelin sheaths. Göthlin (1913) showed that in *Homarus* and *Astacus* the sheaths of the leg nerves are not negatively birefringent, as are the medullary sheaths in prawns and Vertebrates, but positively so, as is the axon of medullated fibres. He made the interesting observation that, after dehydration with glycerine, these nerves became negatively birefringent. His suggestion, that this was due to some orientation in the fatty substances contained in the nerves, has been confirmed by Schmitt, Bear, and Clark (1935). They also showed that, contrary to the

opinion of Boehm (1933) and Schmitt and Wade (1935), much connective tissue is present, as was shown independently by Young (1935, *c*).

In order properly to evaluate the results of investigations of the action potentials, heat production, metabolic exchanges, excitation characteristics, X-ray diffraction patterns and other features of these nerves, further information is needed about the visible structure of the sheaths, the possible presence of any especially fatty layers and the proportion of the nerves which is occupied by the axons.

To obtain information on these points, nerves were removed from the walking legs of *Maia*. The legs were cut open with bone cutters and the nerves carefully dissected out and fixed, stretched to their natural length, on pieces of card. It is very difficult to obtain good fixation, especially of the smaller axons. Rapidly acting fixatives, such as Bouin's fluid or mercuric chloride, produce very serious distortion. Flemming's fluid, made up in sea water and containing very little or no acetic acid, gave better results, but the best were obtained with 1% chromic acid, dissolved in sea water.

The leg nerve is made up of a number of separate bundles, each surrounded by a thin perineurium of collagen fibres. This perineurium is continuous with an endoneurium forming an irregular meshwork of very fine fibrils extending throughout the nerve, nuclei being scattered at intervals. Around the nerve fibres the collagen seems to be thickened, so that each axon runs in a connective tissue tube, very much as in Cephalopods (figs. 14, 15, Plate 15). There are irregular spaces throughout the nerve which stain faintly with red by the azan technique, and are presumably occupied by tissue fluids.

The axons of the leg nerves vary in diameter from less than $1\ \mu$ to $20\ \mu$, but, whereas the sheaths of the smaller fibres are very thin, those of the larger are excessively thick, so that, with its sheath, a fibre whose axon is $15\ \mu$ in diameter may be as much as $80\ \mu$ across. Thus the larger fibres have relatively thicker sheaths than the smaller, this being exactly the opposite of the condition in Cephalopods, and perhaps indicating that, in *Maia*, the sheath plays some definite part in the activity of the axon.

It seems probable that these larger axons run to the muscles, including, perhaps, both excitatory and inhibitory fibres (*see* Biedermann, 1887, Hardy, 1894, Mangold, 1905, Barnes, 1931, Pantin, 1934, and others). There are relatively few of them in the nerve and they usually run in separate bundles, accompanied by few or no smaller fibres. Their numbers and sizes vary at different levels along the leg. Towards the base a few very large fibres, of the type shown in fig. 17, Plate 15, can be seen running quite separate from all other bundles.

The sheaths of these very large fibres have been investigated with especial care, since it was in them that Lullies (1933) claimed to have found "eine besonders deutliche Markscheide". Examination of preparations fixed and stained in a variety of ways has shown that the sheaths consist of concentric sheets of what is, apparently, collagenous connective tissue, continuous with the rest of the endoneurium (figs. 15-18, Plate 15). This tissue stains distinctly with anilin blue, and red with van Gieson's stain, though in neither case is the stain very dark, the fibres being very fine. Nuclei are scattered throughout the thickness of the sheath, and some of them lie in its very innermost layers, often pressed close against the axon. Between the collagenous lamellae are narrow channels, filled with a finely granular substance which stains red with the azan technique, and is presumably tissue lymph.

After fixation with osmium tetroxide or Flemming's fluid, no dense black ring comparable with the medullary sheath of a vertebrate axon can be seen; the sheaths may be somewhat blackened, but not more darkly than the connective tissue in other organs. The collagenous sheets darken more than the lymph between them. After fixation with Flemming's fluid the concentric lamellae are still stainable with anilin blue, a reaction which distinguishes them sharply from vertebrate medullary sheaths.

I have not been able to make any clear distinctions between inner and outer layers of the sheath, such as were found by de Renyi (1929) in the sheaths of the giant fibres of *Homarus*. The innermost layer sometimes has a smoother appearance than the rest, from which it may also separate somewhat after fixation. It is also often rather more darkly stained with osmium tetroxide (fig. 15, Plate 15).^{*} Its nuclei, however, are similar to those elsewhere in the sheath. Although further embryological and chemical investigations are highly desirable, we have sufficient data to conclude that *both the large and small axons of the leg nerves of Maia are enclosed in continuous sheaths, formed of sheets of a collagen-like substance, interspersed with nuclei, containing some fat, and uninterrupted by any breaks comparable with the nodes of Ranvier.* Between the fibrous sheets are spaces filled with lymph, so that, except for the very thin innermost layer, the axon is probably in continuity with the interstitial fluids of the nerve, which in turn is only separated from the outside by a very thin perineurium.

^{*} Drs. F. O. Schmitt and R. S. Bear, in a paper of which they kindly showed me the MS., have demonstrated by the use of polarized light that there is a layer of oriented fatty molecules in this region.

PROPORTION OF THE NERVE OCCUPIED BY AXONS

Accurate estimates of the proportion of the nerves occupied by the axons are very difficult to obtain, because of the difficulty of fixing the smaller axons. Exact measurements would, in any case, be of little value, since the different bundles vary considerably in their make-up. The following estimates were obtained by cutting out and weighing appropriate portions of photographs of transverse sections of nerves fixed in 1% chromic acid dissolved in sea water, embedded in paraffin, sectioned and stained by the azan technique.

In a bundle consisting of small fibres only (less than $5\ \mu$), the axons comprised 65%, the connective tissue and interfibrillary spaces 35% of the cross-sectional area. The bundles of larger fibres contain a much smaller proportion of the axoplasm. Thus a bundle of 7 axons of $7\text{--}15\ \mu$ in diameter contained only 25% of axon in cross-sectional area. In the very large axons at the base of the limb the proportion is even lower. In the case of a fibre which, with its sheath, was $70\ \mu$ in diameter, weighing the photograph showed that only 10% of the cross-sectional area was occupied by axoplasm.

Since these bundles of large fibres occupy only a small portion of the whole nerve, we may estimate that in a preparation of the middle portion of a leg nerve of *Maia*, such as is commonly used for physiological investigation, between 60 and 70% of the cross-sectional area is occupied by axoplasm.

In order to make quantitative comparison between the heat production or metabolic exchanges of *Maia* nerves and those of other animals, we need to know not only what proportion of the nerve is made up of axons, but also whether any nerve cell bodies are present in the trunks. Two nerves were therefore removed from the first walking leg along their whole lengths, folded carefully into bundles 1–2 cm. long, and sectioned serially. Careful search through these preparations failed to show any nerve cell bodies. Such work is very tedious and it is possible that isolated cells were missed, but it may safely be concluded that in these highly developed Arthropods, as in Vertebrates, the great majority of cell bodies are collected into definite ganglia, few or none being scattered along the peripheral nerves.*

* This investigation does not exclude the possibility that there is a network of neurons at the *periphery*, as suggested by Tonner (1933), though I have no reason to believe that such neurons exist.

DISCUSSION

This histological study, although it does not substantiate the claim of Lullies that there are true medullated fibres in the nerve trunks of *Maia*, nevertheless confirms the most important aspect of his work in showing that fibres of different sizes are present (*see also* Barnes, 1931). Even though none of these have true myelin sheaths, yet one would expect, on the analogy of vertebrate nerves, that they would differ in excitation characteristics and conduction velocity. This was shown by Lullies to be so, and he has therefore indicated that the relation between fibre size, excitability, and conduction velocity is in a general way similar in vertebrates and in *Maia*, although the positive identification of his groups of fibres with the B2, B3, and C groups of vertebrates is hardly justified.

The conception of the nerve fibres of these invertebrates, as consisting of a more fluid axoplasm contained within a nucleated sheath, is a partial return to the views held during the last century, before the fibrillar theories of axon structure become dominant. The description of "Nervenröhren", in such works as those of Retzius (1890), more accurately represent the structure of the living fibres than do the accounts of most later workers. Jenkins and Carlson (1904) have shown experimentally that the physiological length of nerves can be altered by stretching and relaxation, as would be expected on this view of their structure.

It was widely known during the last century that axons may be of semi-fluid consistency. Thus Huxley (1880) speaks of the contents of the nerve fibres of *Astacus* as "a fluid of gelatinous consistency", and Hardy (1894) saw granules moving about within large axons of the same animal. More recently Bozler (1927) has shown that the "neuroplasma" which surrounds the neurofibrils visible in the living nerve cells of the medusa *Rhizostoma* is "sehr dünnflüssig". de Renyi (1929) has shown the same for the axoplasm of the giant fibres of *Homarus*. In the frog, however, he believed the axon of medullated nerve fibres to be rather rigid gel.

The disagreements of histologists about the structure of nerve fibres have centred mainly around the question of whether the axons are further internally differentiated into neurofibrils. The literature of this controversy has been reviewed by Parker (1929) and Peterfi (1929). Except in *Rhizostoma* and *Homarus*, definite optically isolable fibrils are not visible in the living state, but numerous workers have reported them as faintly indicated ("swach angedeutet", Ettisch and Jochims, 1926). They become clearer after injury to the fibre, and after appropriate fixation and staining.

The present observations have failed to reveal any definite continuous fibrils within the axon, either in living or fixed preparations, but there is nevertheless a faint visible striation in the axoplasm.

The widespread occurrence of this elusive longitudinal visible striation, together with such facts as the birefringence of the axon (Schmidt, 1934) and the thermal shortening of nerve (Schmitt and Wade, 1935), point very strongly to the conclusion that there is a pattern of micelles oriented along the axon. Change in the state of such a system might be expected readily to produce grosser fibrils by coagulation of the parallel micelles, especially if the change involves dehydration, as do so many histological techniques.

Boehm (1933) and Schmitt, Bear, and Clark (1935) have tried to elucidate this micellar pattern by X-ray diffraction studies. They have provided much interesting information about the structure of the medullary sheath, but have not so far revealed any diffraction pattern which is positively due to the axon. Since they have not been able to examine any preparations consisting exclusively of axoplasm, it is impossible yet to be certain whether there are systems of orientated molecules in the axon capable of giving X-ray diffraction patterns. It is hoped that the giant fibres of Cephalopods, which can be readily isolated and have only thin sheaths, may provide suitable material for investigation of this problem.

SUMMARY

In Cephalopods every axon of the peripheral nerves is covered by a continuous sheath formed of nucleated sheets of a tissue resembling collagenous connective tissue.

The substance of each axon completely fills its sheath; it contains no definite neurofibrils traceable as separate entities, but there is a very faint longitudinal striation visible both in living and fixed fibres. More distinct fibrillae appear if the axon be damaged in any way.

After section of a giant nerve fibre, the axoplasm flows out from the connective tissue tube. The substance of the axon is therefore a rather viscous fluid, the faint visible striation suggesting the presence of longitudinally orientated micelles which, by coagulation, produce the more definite fibrils seen in damaged axons.

65–70% of the cross-sectional area of Cephalopod nerves is occupied by axoplasm, the remainder being connective tissue sheaths, blood vessels, and tissue lymph spaces.

Estimates of the numbers and diameters of the axons in the fin nerve, nerve to the stellate ganglion, and stellar nerves are given.

The leg nerves of *Maia* consist of separate bundles each bounded by a thin perineurium. Each axon is surrounded by a connective tissue sheath which is nucleated, continuous over the whole surface of the axon and containing some fat, although not histologically comparable with the medullary sheath of vertebrates.

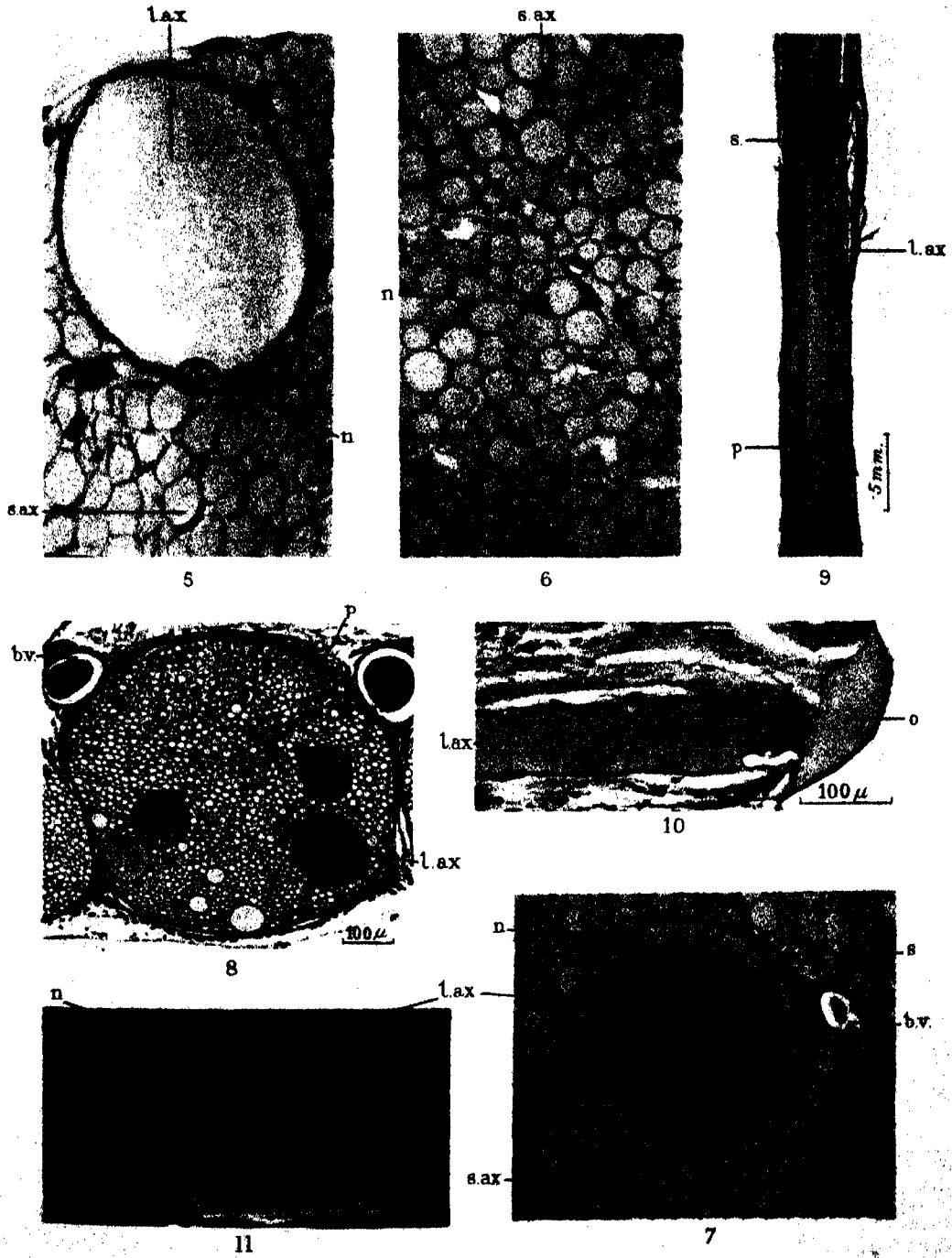
The sheaths around the larger axons of *Maia* are very thick, consisting of many concentric layers, interspersed with tissue lymph spaces.

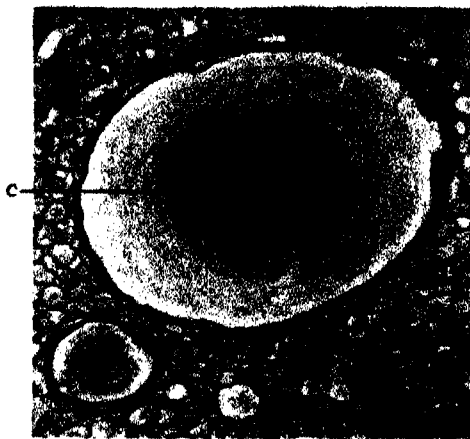
60-70% of the cross-sectional area of the leg nerves of *Maia* is occupied by axoplasm. In the case of the largest fibres, running in separate bundles, as little as 10% of the cross-sectional area is axoplasm, the remainder being sheath.

No nerve cell bodies were found anywhere along the length of leg nerves of *Maia* (ischus to dactylus), by the examination of serial sections.

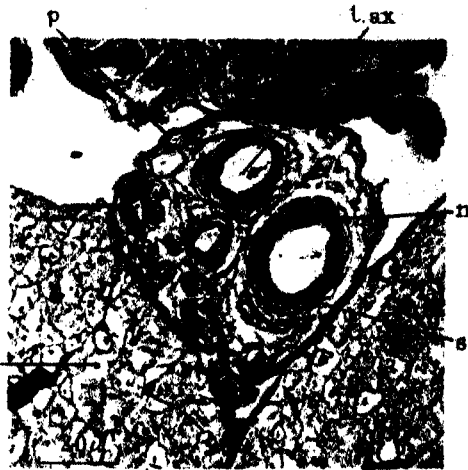
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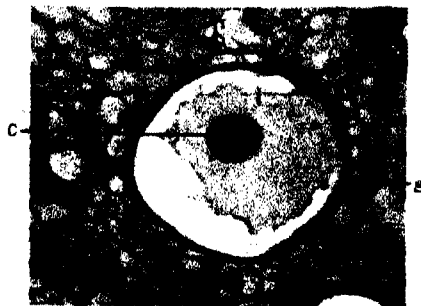




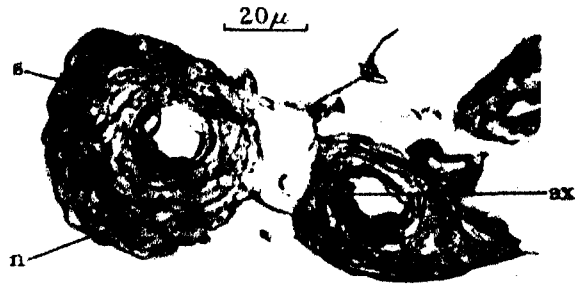
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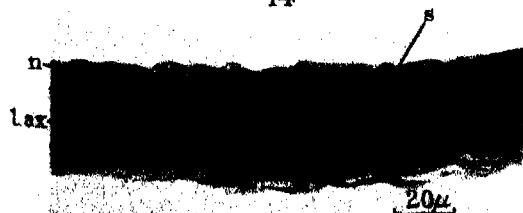
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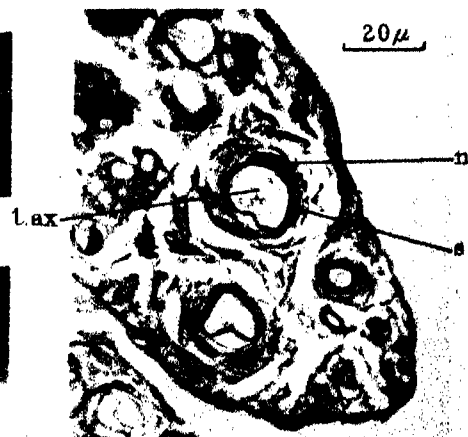
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EXPLANATION OF PLATES

Figs. 5 to 13 are of *Sepia officinalis*, figs. 14 to 18 of *Maia squinado*.

Lettering—*ax.*, shrunken axon; *b.v.*, blood vessel; *c.*, darkly staining core of axon; *l. ax.*, large axon; *n.*, nucleus of sheath; *o.*, substance which has flowed out from cut end of axon; *p.*, perineurium; *s.*, sheath; *s. ax.*, small axon.

PLATE 14

- FIG. 5—Portion of T.S. stellar nerve, showing large and small fibres. Fixed mercuric chloride and acetic acid; stained iron haematoxylin.
- FIG. 6—Portion of T.S. stellar nerve, showing the structure of the smaller fibres. Fixed saturated picric acid in sea water, stain azan.
- FIG. 7—T.S. giant fibre from stellar nerve. Fixation picroformol in sea water, stain azan.
- FIG. 8—T.S. whole stellar nerve. Fixation and staining as fig. 6.
- FIG. 9—L.S. whole stellar nerve, showing a giant fibre. Fixation Bouin's fluid, stain azan.
- FIG. 10—L.S. end of a stellar nerve, cut 15 minutes before fixation. Some of the axoplasm of the giant fibre has flowed out. Fixation potassium bichromate in sea water, stain iron haematoxylin.
- FIG. 11—L.S. giant fibre of *Sepia* in the stellate ganglion. Fixation formaldehyde in sea water, stain Cajal's method.

PLATE 15

- FIG. 12—T.S. giant fibre in stellar nerve to show central core formed as an artefact. Note radial pattern of the fibrillation, as if the axoplasm had been swirled around. Fixation picro-formol in sea water, stain azan.
- FIG. 13—T.S. stellar nerve to show appearance of basophil core as an artefact at centre of a giant fibre. Fixation formaldehyde in sea water, stain iron haematoxylin.
- FIG. 14—Small fibres from leg nerve. Fixed in 2% osmium tetroxide and teased in glycerine.
- FIG. 15—Large fibre from leg nerve, preparation as fig. 14.
- FIG. 16—T.S. portion of leg nerve showing large and small fibres. Fixation 1% chromic acid in sea water, stain azan.
- FIG. 17—T.S. very large nerve fibres from base of leg nerve. Fixation 0.5% osmium tetroxide in sea water, no further staining.
- FIG. 18—T.S. portion of leg nerve to show fibres of medium size. Fixation 1% chromic acid, stain iron haematoxylin.
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The Effect of Fumarate on Respiration

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INTRODUCTION

The recent work of Szent-Gyorgyi and his co-workers (Gozsy and Szent-Gyorgyi, 1934; Annau *et al.*, 1935) on the importance of fumarate in cellular respiration assigns a definite and fundamental function to the di-carboxylic four-carbon-atom acids and to the enzymes which activate them. Essentially their view is as follows: the reversible system, fumarate-oxalacetate, functions catalytically in transporting activated hydrogen from the metabolite dehydrogenation systems to the cytochrome (Warburg-Keilin) system through the medium of an intermediary substance. The oxalacetate formed takes up hydrogen, going either to fumarate, or to succinate by "over-reduction". The succinate thus formed is converted to fumarate by means of succinodehydrogenase, and the catalytic cycle is completed.

Malonate is known to inhibit succinodehydrogenase (Quastel and Wooldridge, 1928), and malonate also inhibits respiration. Szent-Gyorgyi *et al.* explain this inhibition by the fact that the succinate normally formed is prevented by malonate from being oxidized to fumarate, and respiration fails owing to a lack of fumarate. They further attribute the decrease in respiration of muscle tissue *in vitro* to a loss of fumarate through diffusion. If, however, this loss is compensated for by the addition of fresh fumarate, respiration proceeds normally. The addition of fumarate therefore "preserves" the normal respiration of the tissue.

The theory of Szent-Gyorgyi *et al.* was based on manometric experiments and on the chemical estimations of fumarate, oxalacetate, and succinate. The results of their manometric experiments have shown that the addition of fumarate increased the respiration of the tissues while that of malonate inhibited it, and fumarate plus malonate showed the same effect as fumarate alone. This has recently been confirmed by Boyland and Boyland (1936) and by Greville (1936). By themselves these results gave no definite answer to the question whether fumarate added to the tissue acted as a catalyst or simply as a substrate. It was

mainly in the chemical estimations of fumaric, oxalacetic, and succinic acids that Szent-Gyorgyi *et al.* found the strongest support for their views. Owing, however, to the great difficulties connected with the correct estimation of these substances in tissue, it seemed to us essential to test the theory by some other method. We have found that this can be done by a study of the kinetics of tissue respiration using the manometric method, provided the experiments are carried out in a suitable medium and properly controlled. The main object of this paper consists, therefore, in the study of the effect of fumarate and of other allied substances on the respiration of muscle tissue.

METHODS AND MATERIAL

The oxygen uptake of pigeon breast muscle or of pig heart muscle was measured in the usual way (Dixon, 1934) in Barcroft differential manometers at 39° C. for periods varying from 90 to 180 minutes. The pigeons were killed by "breaking the neck". The breast muscle was removed immediately, cooled on ice for one-half minute, and ground in a Latapi mincer, the second disk of which had been removed to minimize damage to the tissue (Szent-Gyorgyi *et al.*, 1935). Small amounts of tissue were weighed on cellophane on a torsion balance and added directly to the flasks. This method was found more accurate than pipetting suspensions of the tissue. The volume of fluid in the respiration flask was always 3 cc., and the amount of tissue approximately 45 mg. dry weight. The amount has, however, been varied from 20 to 60 mg. without change in the observed Q_0 , (c.mm. O_2 absorbed per hour per mg. dry weight of tissue). Measurements of the respiratory quotient, R.Q., were made in differential manometers, the method recommended by Dixon (1934) being used.

The conclusions in this paper are based on a large number of experiments (90 pigeons and 46 pig hearts). Considerable variation in the Q_0 , of both pigeon breast muscle and of pig heart muscle has been observed from animal to animal. Because of this variation it was essential that all experiments be adequately controlled. Every group of our experiments, therefore, contained a control determination, run on the same tissue at the same time as the experiment itself, and each experimental result was compared with its own control. All figures in this paper are for pigeon breast muscle and are of representative, individual experiments; they are not to be read diagrammatically.

THE EFFECT OF FUMARATE AND MALONATE

Our first experiments were made with pigeon breast muscle using M/15 phosphate buffer according to the procedure of Szent-Gyorgyi *et al.* (1935). Fig. 1* confirms their observation that malonate inhibits respiration, fumarate increases it, and fumarate plus malonate increases

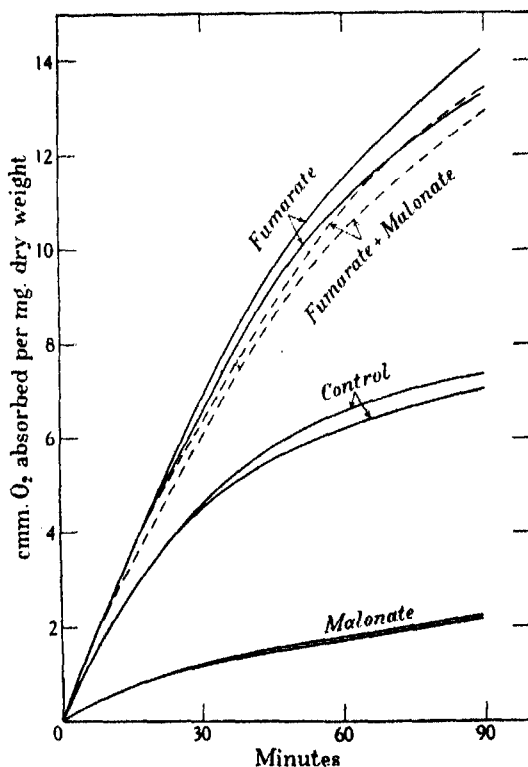


FIG. 1—The effect of fumarate and of malonate on the respiration of pigeon breast muscle. M/15 phosphate buffer. Fumarate and malonate in concentration of 0.0066 M. each.

* There appears to be no convenient term in the literature which indicates the amount of oxygen consumed by a unit of tissue at any given time. The " Q_{O_2} ," at any given time is a measure of the rate rather than the amount of oxygen uptake and is not suitable for plotting the curve of oxygen consumption directly. We have therefore expressed our results, and particularly our graphs, in terms of oxygen uptake, U_{O_2} , that is, cubic mm. O_2 absorbed per mg. dry weight of tissue in the time t . U_{O_2} values can be converted to Q_{O_2} values by the following formula:

$$Q_{O_2} = \frac{U_{O_2} \times 60 \text{ minutes}}{t \text{ minutes}}$$

from which it follows that at 60 minutes the U_{O_2} is numerically equal to the Q_{O_2} .

it, though somewhat less than fumarate alone. In 24 experiments the average oxygen uptake expressed in U_{O_2} * (c.mm. O_2 consumed per mg. dry weight) at 90 minutes was 9.5 for the control tissue, 13.0 in 0.0066 M. fumarate, 3.5 in 0.0066 M. malonate, and 11.5 when both fumarate and malonate were present.

When the respiration of the control tissue varied markedly from the normal, variable results were obtained, except for the consistency of malonate inhibition. In five cases with a control oxygen uptake (U_{O_2}) of 14.2 instead of 9.5 at 90 minutes, the increase due to fumarate was only 15% instead of 37%. The assumption was that relatively high amounts of fumarate were already present in these tissues. When the control U_{O_2} was low, as in stored tissue, fumarate addition failed to raise the respiration. Such tissues were probably lacking in some essential factor other than fumarate, since they usually responded to fumarate on the addition of muscle extract.

The general fumarate-malonate effect was also observed with pig heart muscle. In 21 experiments in M/15 phosphate buffer the U_{O_2} for 90 minutes averaged 6.4 for control muscle, 9.2 in the presence of 0.0066 M. fumarate, 3.9 with 0.0066 M. malonate, and 6.9 with the two together. Similar effects were obtained with rat liver and with pigeon liver.

THE EFFECT OF TISSUE EXTRACT

Tissue extract increased the respiration of muscle in phosphate buffer to a much greater extent than fumarate: 300% in the 60 minute Q_{O_2} , as compared with a maximum of 50% obtained with fumarate. The extract was prepared as follows: minced pigeon breast muscle was suspended in an equal weight of water and stirred at intervals for one hour. The extract was squeezed through muslin, heated to 90° C. until coagulation was complete, and then filtered. The filtrate was stored at 0° C. and retained its activity for several weeks, unless infected.

Maximum increases in respiration were obtained with 1.0 to 1.5 cc. of this extract. For smaller amounts of extract the increase was roughly proportional to the amount of extract added. The percentage increase due to large amounts of extract was essentially the same for both the initial and final Q_{O_2} values; in other words, the extract had increased rather than maintained the original rate of oxygen uptake. The respiratory quotient of this increased respiration was normal (*see below*).

When fumarate plus extract was added, the initial effect was not much greater than that of extract alone. After the first hour, however, the fumarate tended to preserve the respiration. Tissues which showed only

a small increase in respiration on addition of fumarate, showed a much greater one on the addition of fumarate plus extract, even though the amount of extract was too small to produce an effect by itself (solid lines, fig. 2).

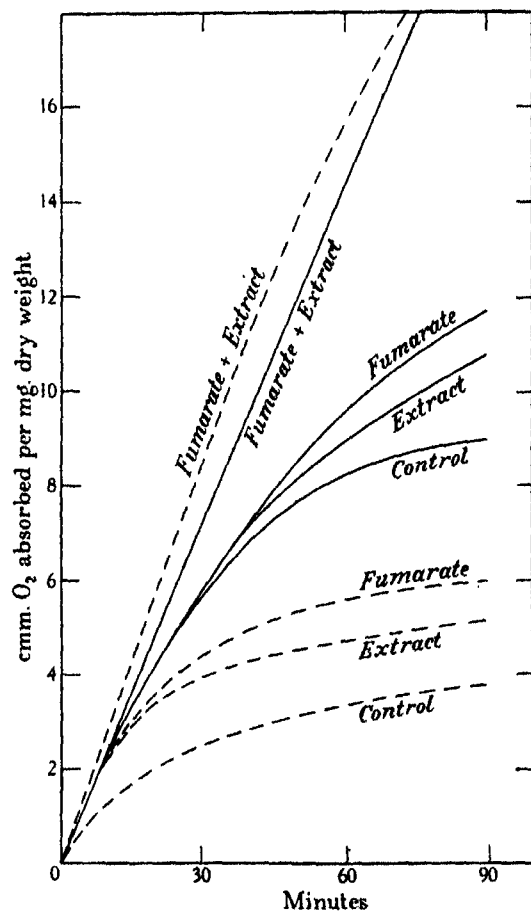


FIG. 2—The effect of grinding on the response of pigeon breast muscle to fumarate and to muscle extract. M/15 phosphate buffer, 0.02 M. fumarate, $\frac{1}{2}$ cc. extract. Broken lines = tissue ground with sand; solid lines = unground tissue.

This extract effect was most strikingly demonstrated with tissue which had been ground with an equal weight of sand, and then suspended in M/15 phosphate. Such tissue had a much lower Q_0 , than unground tissue ($Q_0 = 2.8$ instead of 7.8 , average of 20 experiments). As in unground muscle, large amounts of muscle extract increased the respiration markedly. Either fumarate alone, or small amounts of extract alone, showed a comparatively small increase in respiration (Table I), but

when the two were added together, the increase was enormous, and the oxygen uptake equalled that of unground tissue similarly fortified, fig. 2. The comparative effect of the combined supplements was thus markedly increased in ground as compared with unground tissue; furthermore, the extract plus fumarate had compensated for the loss in respiration due to the destruction of the cells themselves.

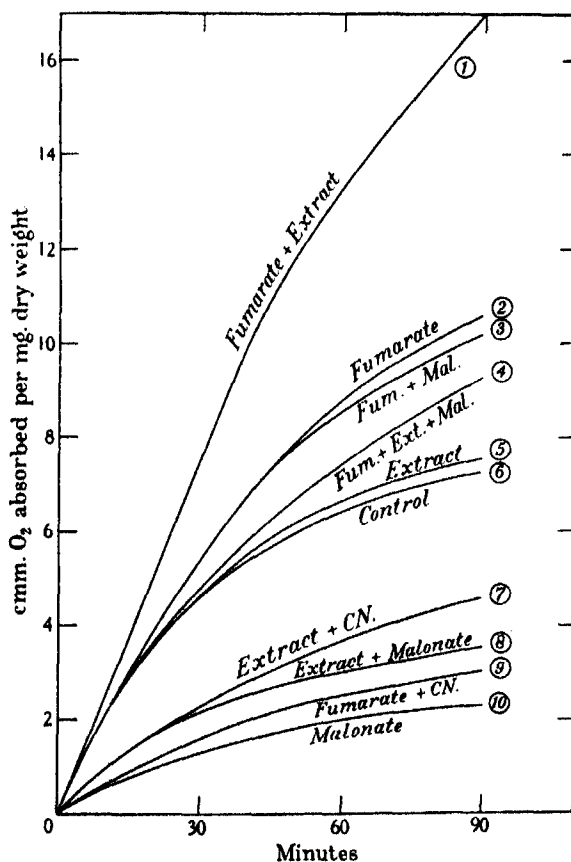


FIG. 3—The effect of inhibitors on the response of pigeon breast muscle to fumarate and extract. M/15 phosphate buffer, 0.02 M., fumarate, 0.02 M.; malonate, 0.003 M.; cyanide, $\frac{1}{2}$ cc. extract.

The stimulating effect of extract was inhibited by malonate. With extract plus fumarate, malonate also inhibited, but to a less extent than with extract alone (fig. 3, curves 4 and 8). That the extract factor was not fumarate was indicated by the malonate inhibition, and furthermore by the fact that increased concentrations of fumarate did not duplicate the effect of extract. Malonate inhibition suggested that the active

agent might be succinate. However, the R.Q. of muscle stimulated by extract was 0.90, whereas succinate in sufficient amounts to duplicate the extract effect lowered the R.Q. to values around 0.5. Furthermore, fumarate plus succinate stimulated respiration much less than fumarate plus extract.

Active extracts have been prepared from yeast, pig heart, and ox heart as well as from pigeon breast muscle. The active factor was dialysable and stable at 90° for 30 minutes. Preparations of co-zymase and of co-ferment showed a similar qualitative action to the extracts, but were quantitatively less active. The extract effect was probably not due to added substrate, as the amounts added were small, and neither glucose nor hexose-di-phosphate increased respiration. Cyanide always inhibited respiration, whether fumarate, extract, or fumarate plus extract were present. Cyanide inhibition was of the same order of magnitude, as found by Dixon and Elliott (1929) in the presence of large amounts of phosphate.

THE EFFECT OF BUFFERS

Phosphate buffer* of M/15 concentration is an abnormal medium for the study of the respiration of surviving tissue as the solution is neither iso-tonic nor complete in inorganic factors. We accordingly extended our observations to Ringer's solution and to a more physiological medium, Krebs's Ringer-phosphate solution,† in which the concentration of phosphate ion is M/60.

In unbuffered Ringer solution the oxygen uptake of muscle was low ($Q_{O_2} = 1$); fumarate failed to increase respiration, and malonate failed to inhibit it, which observation agreed with that of Szent-Gyorgyi (Annau *et al.*, 1935). This low respiration could be attributed to a rapid increase in the acidity of the system and also possibly to a deficiency in necessary phosphate.

Ringer solution containing M/60 phosphate was found to be a satisfactory medium for experiments with muscle tissue. The Q_{O_2} was raised from 7.8 in M/15 phosphate to 9.6 in Ringer-phosphate (average

* 2/15 M. phosphate buffer made as follows: 70 cc. of M/4 Na_2HPO_4 + 30 cc. of M/4 KH_2PO_4 + 87.5 cc. H_2O . 1½ cc. of this was added to each flask and diluted to M/15 with 1½ cc. of water or of the solution to be tested.

† Krebs's Ringer-phosphate made as follows: 100 parts 0.9% NaCl, 4 parts 1.15% KCl, 1 part 2.11% KH_2PO_4 , 1 part 3.82% $MgSO_4$, 21 parts of a mixture of 40 cc. M/4 Na_2HPO_4 + 2 cc. N/1 HCl. This solution was made up in double concentration, 1½ cc. added to each flask and then diluted with 1½ cc. of H_2O or of the solution to be tested.

of 24 experiments), and the sensitivity of the tissue to fumarate was markedly increased (fig. 4, curves 1, 2, 4, and 6). In Ringer-phosphate medium the oxygen uptake of pigeon breast muscle was often constant for over 90 minutes provided fumarate was added to the system.

The compensating effect of fumarate for malonate inhibition differed with the two media. In M/15 phosphate, equimolecular quantities of

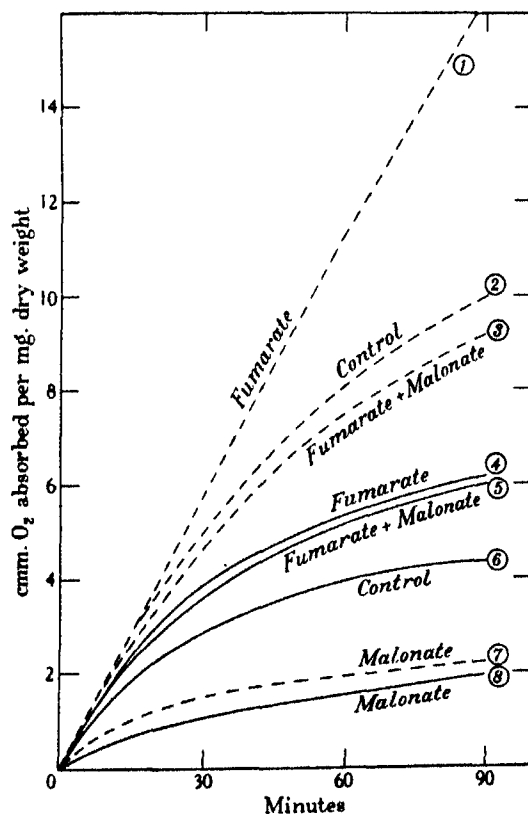


FIG. 4—The effect of the medium on the response of pigeon breast muscle to fumarate and malonate. 0.02 M. fumarate, 0.02 M. malonate; broken lines = Ringer-phosphate buffer; solid lines = M/15 phosphate buffer.

fumarate and malonate added to pigeon breast muscle showed nearly the same effect as fumarate alone. Fumarate sometimes compensated completely for malonate inhibition though with no degree of regularity. In Ringer-phosphate, however, fumarate plus malonate was much less effective in increasing respiration than fumarate alone, fig. 4. Similar results had been obtained in phosphate buffer supplemented with muscle extract, fig. 3. The explanation probably lay in the fact that in phosphate

buffer the tissue was not respiring at an optimum rate, even in the presence of fumarate, and a slight malonate, inhibition was masked by the failure of other elements in the system. In Ringer-phosphate, the tissue was respiring at a more nearly optimum rate, and the true malonate effect could be observed.

Tissue was found to be more stable in Ringer-phosphate than in plain phosphate buffer. Tissue stored at 0° C. for periods of one-half to one

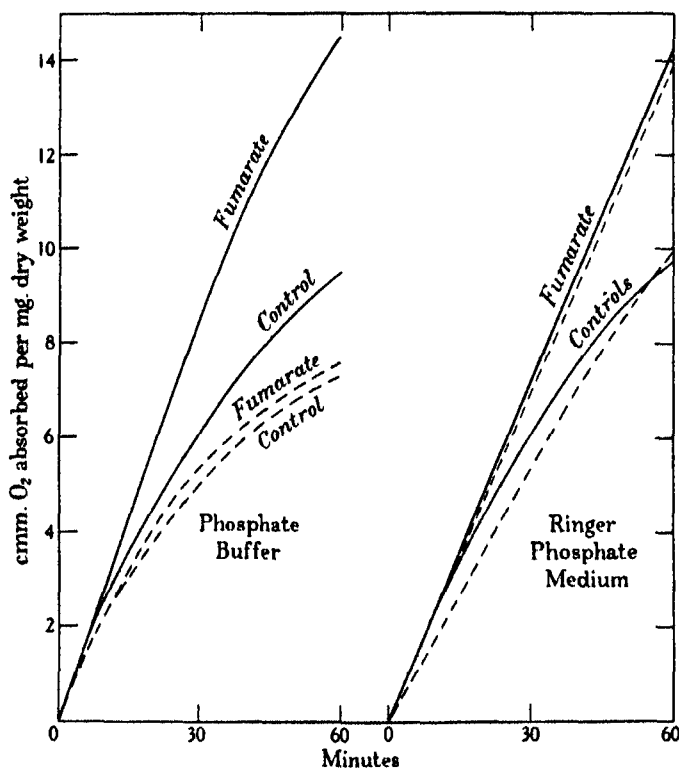


FIG. 5—The effect of the medium and of the "age" of the tissue on the fumarate response in pigeon breast muscle. Broken lines = muscle stored (aged) at 0° C for 30 minutes; solid lines = fresh muscle.

hour before use showed both a decreased respiration and a decreased sensitivity to fumarate when measured in plain phosphate buffer. Thirty to forty minutes after the tissue was removed from the body practically no fumarate response was obtained, fig. 5. This emphasizes the importance of the "age" of tissue when one is measuring respiration in phosphate buffer. In Ringer-phosphate buffer, on the contrary, respiration was unimpaired after the tissue had stood for one-half hour at 0° C., and the fumarate response was practically the same as in fresh tissue.

Pig heart muscle, like pigeon breast muscle, showed a greater oxygen uptake, and a greater sensitivity to fumarate in Ringer-phosphate, or in phosphate plus extract, as compared with plain phosphate buffer.

The differences in the two media were not due to differences in p_H , as the buffering capacity of the M/60 phosphate was found to be adequate. The inorganic ions in the Ringer solution may have been responsible for the improved sensitivity of the tissue to fumarate. As, however, phosphate plus muscle extract behaved similarly to Ringer-phosphate, the possibility of essential organic factors could not be excluded. The explanation probably lay in the preservation of cellular integrity in Ringer-phosphate, whereas in M/15 phosphate, which was neither isotonic nor complete in inorganic ions, the cells rapidly deteriorated, permitting the loss of necessary substances by diffusion. The addition of fumarate and muscle extract apparently restored these substances to the system.

THE "PRESERVING" ACTION OF FUMARATE ON RESPIRATION

When added to muscle, fumarate does not increase the initial rate of respiration, but tends rather to maintain it. This has been suggested by the experiments of Szent-Gyorgyi *et al.*, who used suspensions of muscle in phosphate buffer. However, neither consistent nor clear cut results are obtained with this medium. In Ringer-phosphate, on the contrary, or in phosphate plus muscle extract, a satisfactory demonstration of the preserving action of fumarate is possible.

Using the latter media, we have added fumarate to muscle from Keilin cups at the beginning of the experiment, or after 20 or 40 minutes, and in every case, the curve of oxygen uptake after fumarate addition continued as a tangent to the control curve, figs. 6 and 7. In other words, the rate of oxygen uptake at the moment of fumarate addition had been maintained. In plain phosphate there was some preserving action of fumarate at 0 or 20 minutes, but none at 40 minutes, fig. 6.

THE EFFECT OF FUMARATE CONCENTRATION

Concentrations of fumarate from 0.0001 M. to 0.02 M. in Ringer-phosphate buffer and from 0.0008 M. to 0.05 M. in phosphate buffer have been studied. Within that range the Q_{O_2} values at 60 minutes were nearly the same for all concentrations of fumarate, the 90-minute U_{O_2} values were slightly lower for concentrations below 0.0004 M., and the 120-minute U_{O_2} values were lower for concentrations below 0.0033 M. Concentrations above 0.05 molar generally showed a slight inhibition.

This indicates that for the first 60 to 90 minutes small concentrations of fumarate stimulated respiration to about the same extent as large concentrations, but that after this initial increase larger amounts of fumarate gave a greater respiration (Table II).

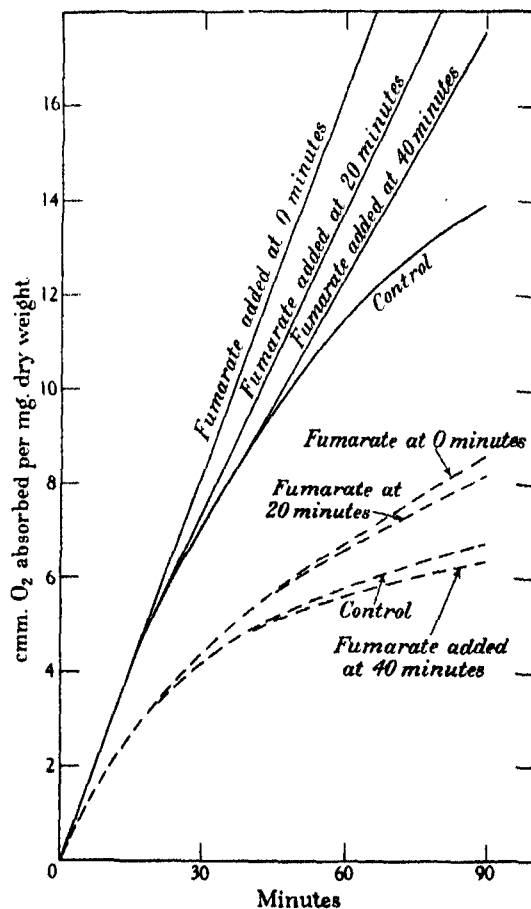


FIG. 6.—The conserving action of fumarate: the delayed addition of fumarate to pigeon breast muscle in phosphate (dotted lines) and in Ringer-phosphate (solid lines). Concentration of fumarate = 0.02 M.

THE EFFECT OF FUMARATE ON THE RESPIRATORY QUOTIENT

The R.Q. of muscle was studied in an attempt to determine the fate of added fumarate, since several paths of fumarate oxidation are theoretically possible. If fumarate functioned only as a catalyst, no change in R.Q. should have been observed when it was added. If the fumarate had been oxidized to oxalacetate, and remained as such, there would have been an

increase in oxygen uptake with no increase in CO_2 output, and the R.Q. would have decreased. If fumarate had been oxidized to oxalacetate and then decarboxylated to pyruvate, the R.Q. would have risen markedly since the theoretical R.Q. for these reactions is 2.00. Finally, if fumarate had been completely oxidized as a substrate, the R.Q. would have increased since the theoretical R.Q. for fumarate oxidation is 1.33.

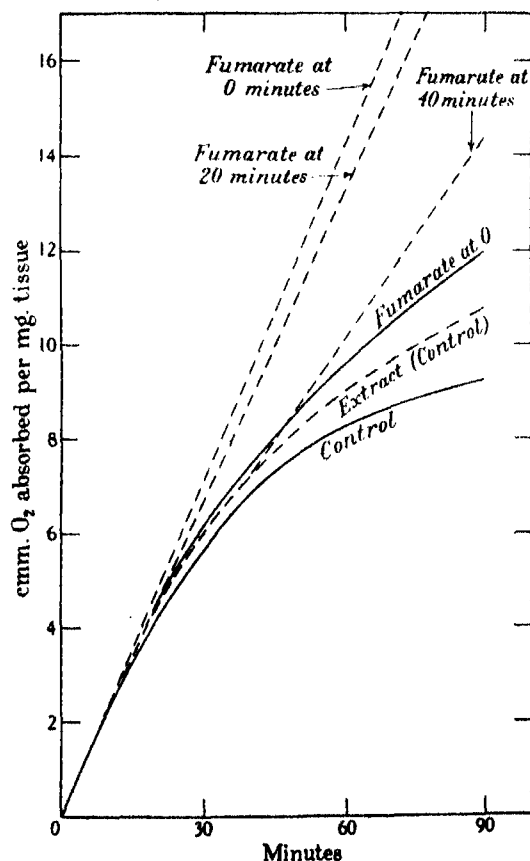


FIG. 7.—The conserving action of fumarate: the delayed addition of fumarate to pigeon breast muscle in phosphate buffer + extract. 0.02 M. fumarate, $\frac{1}{2}$ cc. extract; broken lines = phosphate + extract; solid lines = plain phosphate.

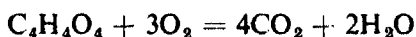
The R.Q. of muscle was studied in both plain phosphate and in Ringer-phosphate medium, supplemented with various amounts of fumarate or allied substances, or of muscle extract. The addition of fumarate increased the R.Q. of muscle from 0.90 to from 1.00 to 1.20, depending upon the amount of fumarate added (Table III). The smaller amounts of fumarate had comparatively little effect on the R.Q., even though the oxygen uptake of the system had been markedly increased.

The inference is that high concentrations of fumarate can function as a substrate, though lower amounts may act catalytically, a conclusion also suggested by the fact that large amounts of fumarate increase respiration more than small amounts (Table II). Muscle extract did not change the R.Q. either of ground or unground muscle, but high concentrations of succinate, as expected, lowered it markedly. The R.Q. did not change during the course of the experiment.

THE CATALYTIC ACTION OF FUMARATE

The concentrations of fumarate sufficient to preserve the respiration of muscle in Ringer-phosphate were surprisingly small, and the increases in oxygen uptake observed were several times greater than could be accounted for by the complete oxidation of fumarate to oxalacetate, or even to CO_2 and H_2O (Table IV).

The theoretical oxygen requirement of the fumarate was calculated as follows:



1.L. of 1 mol. fumarate = 3 mol. O_2 = 67.2 L. O_2

1.L. of 0.001 M. fumarate = 67,200 c.mm. O_2 .

3 cc. of 0.0002 M. fumarate requires 40 c.mm. O_2 .

Therefore the increase in oxygen uptake which would be obtained from the complete oxidation of 3 cc. of 0.0002 M. fumarate is 40 c.mm. For the conversion of this amount of fumarate to oxalacetate only one-sixth of this amount, or 6.6 c.mm. O_2 , would be necessary.

Concentrations of fumarate up to 0.0016 M. raised the respiration of the tissue more than could be accounted for by the complete oxidation of the fumarate itself. The increased respiration in the presence of 0.0002 M. fumarate was approximately five times that which could be attributed to its action as a substrate. The presumption is that fumarate, or some natural derivative of fumarate, functions catalytically.

If fumarate functions as a catalyst, however, it should be possible to detect its presence in the reaction mixture at the end of an experiment. This we have done manometrically. Varying amounts of fumarate were added to pigeon breast muscle in Ringer-phosphate solution and allowed to respire for two hours at 39° C. The mixture was then coagulated by heating at 90° C. and filtered. Aliquots of these filtrates, "fumarate juice", were then added to fresh samples of breast muscle in Ringer-phosphate in amounts equivalent to 0.1 mg. or 0.2 mg. original fumarate. Three types of controls were run:

(1) unsupplemented controls; (2) aliquots of "control juice", that is the filtrate from experiments to which no fumarate had been added; (3) aliquots of "control juice" plus 0.1 mg. fresh fumarate. The latter was considered necessary to demonstrate that the tissues were capable of responding to fumarate in the presence of used juice.

In all cases "fumarate juice" gave higher respiration values than "control juice", that is, some factor stimulating respiration was present in the juices to which fumarate had been added, after two hours of respiration, even though the increased respiration of such mixtures had been sufficient to oxidize completely the added fumarate, fig. 8.

With this method, the recovery of "fumarate" averaged only about 50%. Control juice sometimes slightly inhibited respiration as compared with unsupplemented controls. Fumarate juice always showed higher respiration than control juice, but frequently not so high as control juice plus fresh fumarate in equivalent amounts.

Two explanations for the incomplete recovery of fumarate activity were considered by us. The first is that a fraction of the fumarate added had been destroyed. However, the amounts chosen, 0.4 to 0.8 mg. sodium fumarate in the experiments from which the fumarate juice had been prepared, were such that the increase in respiration was more than could be accounted for by the complete oxidation of the added salt. Thus even if some fumarate had been destroyed, the remainder must still have acted catalytically. Another explanation is that the recovery of "fumarate" was incomplete by our technique. Losses due to adherence of fumarate to the protein coagulum are possible, and inhibiting substances may have been present in the used juices in an unequal degree. In spite of these difficulties, fumarate juice gave consistently higher respiration values than control juice, which is what one would expect to observe, if fumarate were still present in the solutions.

OTHER 4-CARBON COMPOUNDS

In Ringer-phosphate not only fumarate, but succinate, oxalacetate, and *d-l* and *l* malate, increased respiration in concentrations of 0.0001 M. or more (Table IV). The respiration increases with these acids were of the same order of magnitude as with fumarate, that is the increase in oxygen uptake in each case was greater than the theoretical requirement of oxygen for the *complete* oxidation of the acids added. The R.Q. of the increased respiration was normal in all cases. These four carbon acids, therefore, behaved like fumarate in all essential particulars. Maleic acid

inhibited respiration in low concentrations, the order of inhibition being the same as for malonate.

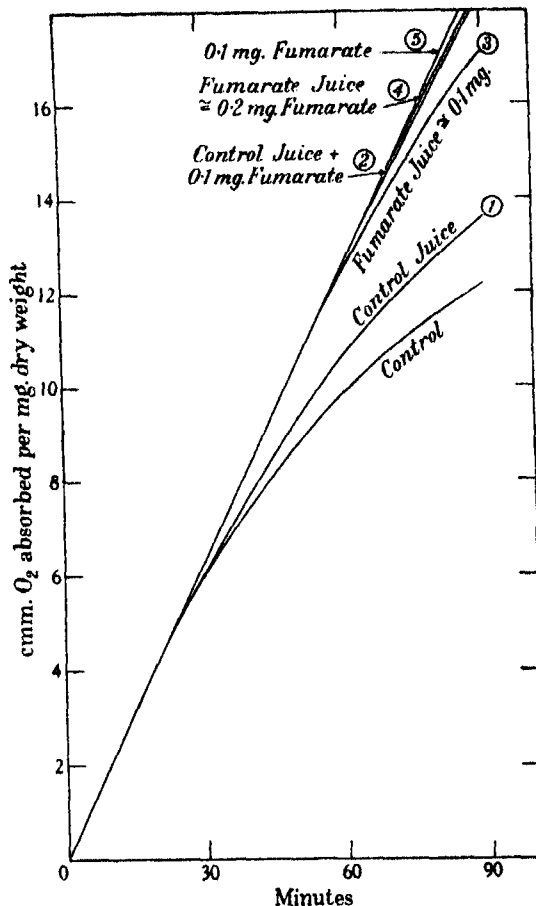


FIG. 8—The recovery of fumarate from "used juice". Pigeon breast muscle in Ringer-phosphate buffer, supplements as follows:—(1) 0.4 cc. control juice (medium in which 200 mg. muscle had been respiring for 2 hours). (2) 0.4 cc. control juice + 0.1 mg. fumarate. (3) 0.4 cc. fumarate juice (medium originally containing 0.0016 M. fumarate, in which 200 mg. of muscle had been respiring for 2 hours; 0.4 cc. is equivalent to 0.1 mg. fumarate originally present). (4) 0.8 cc. fumarate juice, equivalent to 0.2 mg. fumarate originally present. (5) 0.1 mg. fresh fumarate.

DISCUSSION

The most striking observation of this series of experiments is the very small amount of fumarate necessary to stimulate the respiration of muscle in a suitable suspension medium; 0.0001 M., or 33 gamma of

TABLE I—THE EFFECT OF FUMARATE AND OF MUSCLE EXTRACT ON THE RESPIRATION OF GROUND PIGEON BREAST MUSCLE*

C.mm. O₂ absorbed in 90 minutes per mg. dry weight. (U₀, 90 minutes)

Control (U ₀ , 90 min.)	Fumarate 0·0066M.~ 0·02 M. (U ₀ , 90 min.)	Extract 0·05-0·25 cc. (U ₀ , 90 min.)	Extract 0·05-0·25 cc. + fumarate (U ₀ , 90 min.)	Extract 1·0-1·5 cc. (U ₀ , 90 min.)	Extract 1·0-1·5 cc. + fumarate (U ₀ , 90 min.)
3·76	6·12	—	—	23·6	—
1·46	4·38	—	—	15·0	26·2
2·05	3·73	—	—	14·0	18·2
2·20	2·87	—	—	14·6	22·2
2·4	4·4	—	—	16·3	21·0
3·3	4·6	5·2	21·0	11·6	22·9
2·1	4·2	4·1	7·5	—	—
2·1	4·2	4·2	11·2	—	—
2·1	4·2	5·2	10·4	—	—
2·8	5·0	6·7	14·4	—	—
2·9	5·9	3·6	7·6	—	—
3·9	—	—	5·3	25·6	35·0
1·5	3·5	4·0	11·0	—	—

* All experiments done in M/15 phosphate buffer.

TABLE II—THE EFFECT OF VARYING AMOUNTS OF FUMARATE ON THE RESPIRATION OF PIGEON BREAST MUSCLE*

Conc. of fumarate	No. of expts.	Control U ₀ , 120 min.		Experiment U ₀ , 120 min.		% Increase
		Average	Range	Average	Range	
0·0001 M.	2	13·6	(13·0-14·2)	17·6	(15·2-20·0)	29
0·0002 M.	9	13·6	(9·4-21·2)	17·7	(12·0-22·6)	30
0·0004 M.	2	10·1	(9·4-10·9)	14·7	(14·3-15·1)	45
0·0008 M.	7	13·6	(9·4-21·2)	18·9	(15·5-21·4)	39
0·0016 M.	5	11·9	(9·4-14·0)	20·0	(17·5-24·5)	68
0·0033 M.	3	10·9	(9·4-11·7)	22·8	(19·0-25·0)	109
0·0066 M.	2	13·0	(11·7-14·2)	26·5	(26-27)	104
0·0133 M.	3	12·5	(11·7-14·2)	25·6	(23·4-29·7)	104

* All experiments done in Ringer-phosphate medium.

TABLE III—THE EFFECT OF FUMARATE AND OF ALLIED SUBSTANCES ON THE RESPIRATORY QUOTIENT OF MUSCLE

Salt added	R.Q.	Control R.Q.	Remarks
<i>Pigeon Breast Muscle</i>			
0.0066 M. fumarate	0.97	0.88	Phosphate buffer, 90 minutes
0.026 M. "	1.07	—	" 90 "
0.026 M. "	1.26	1.02	" 30 "
0.026 M. "	1.01	0.93	Phosphate buffer + extract (ground muscle) 60 minutes
0.02 M. "	1.2	—	Ringer-phosphate, 30 minutes
0.02 M. "	1.2	0.91	" 60 "
0.0008 M. "	0.98	0.89	" 90 "
0.0002 M. succinate	1.07	0.94	" 90 "
0.0008 M. "	1.01	—	" 90 "
0.02 M. "	0.53	0.96	" 90 "
0.0016 M. malate	1.00	—	" 90 "
0.0008 M. oxalacetate ..	1.00	0.92	" 90 "
<i>Pig Heart Muscle</i>			
0.0066 M. fumarate	0.95	0.85	Phosphate buffer, 60 minutes
0.02 M. succinate	0.43	0.90	" 60 "
		0.90	Phosphate buffer + muscle extract, 60 minutes
		0.90	Phosphate buffer + yeast extract, 60 minutes
		0.88	Ringer-phosphate, 60 minutes

fumaric acid added to 200 mg. of muscle in 3 cc. of medium. With this amount of fumarate the increase is 6 to 10 times more than could be accounted for by the complete oxidation of the added fumarate. Concentrations of fumarate up to 0.0016 M. generally produced an increase of oxygen consumption greater than the theoretical required for its complete oxidation. Such results strongly indicate catalytic action.

Another argument in favour of the view that fumarate acts catalytically is the observation that solutions to which small amounts of fumarate had been added retained their activity after prolonged contact with respiring tissues. The "used juices", that is the medium in which tissues had respired for a period of two hours, increased the respiration of fresh tissue provided fumarate had originally been added to them. The used juices from the control experiments to which no fumarate had been added were without effect. When the original fumarate concentration was 0.0016 M., and only 0.4 cc. of used juice (equivalent to 0.1 mg. fresh

TABLE IV—THE INCREASE IN THE RESPIRATION OF PIGEON BREAST MUSCLE STIMULATED BY SMALL AMOUNTS OF FUMARATE AND ALLIED SUBSTANCES*

Concentrations	No. of Expts.	Oxygen uptake			
		Observed increase†		Theor. increase‡	
		Range	Average	Complete oxidation	Oxidation to oxalacetate
		c.mm. O ₂	c.mm. O ₂	c.mm. O ₂	c.mm. O ₂
0·0001 M. fumarate	2	82–221	151	20	3·3
0·0002 M. „	8	148–392	235	40	6·6
0·0004 M. „	2	256–267	261	80	13·3
0·0008 M. „	8	208–392	268	160	26·6
0·0016 M. „	6	184–482	308	320	53·3
0·0001 M. succinate	1	—	201	23	6·6
0·0002 M. „	2	123–197	160	47	13·3
0·0008 M. „	3	106–430	267	187	53·3
0·0001 M. malate	1	—	90	20	3·3
0·0002 M. „	1	—	140	40	6·6
0·0008 M. „	3	151–201	182	160	26·6
0·0016 M. „	2	323–373	348	320	53·3
0·0001 M. oxalacetate	1	—	238	16·6	—
0·0002 M. „	2	111–271	191	33·3	—
0·0008 M. „	2	206–318	262	133·3	—

* All experiments done in Ringer-phosphate medium.

† Increase calculated as follows: $U_{O_2}^{120''}$ of experimental sample — $U_{O_2}^{180''}$ of control sample $\times$ dry weight of tissue.

‡ Calculations given in test.

sodium fumarate) was added to fresh tissue, approximately 50 to 70% of the “fumarate activity” was recovered.

When small amounts of fumarate are added to muscle, the R.Q. of the respiring system is changed very little, even though the amount of oxygen uptake has been greatly increased. This is what one would expect to observe, if fumarate acted catalytically.

Not only fumarate, but its natural derivatives succinate, oxalacetate, and malate, increased respiration in the same concentrations, and in the same degree as fumarate; and, furthermore, the R.Q. was the same as when fumarate was added. Since these substances are all interconvertible in the presence of tissue, the observations are in harmony with the results obtained with fumarate

The fact that in small concentrations fumarate acts in a catalytic manner

does not imply that fumarate functions only as a catalyst. With increased concentrations of fumarate a significant increase in the R.Q. value was obtained. Furthermore, higher amounts of fumarate increased respiration over a long period (two hours) more than low amounts. Apparently some of the added fumarate is oxidized in the presence of respiring tissue, particularly when the concentration of fumarate exceeds 0.0016 M. We are of the opinion that fumarate acts primarily as a catalyst, but that in higher concentrations it may also act as a substrate.

Fumarate does not increase the rate of respiration, but tends rather to stabilize it. In suitable media such as Ringer-phosphate solution, or phosphate plus muscle extract, the rate of respiration remained constant after the addition of fumarate. If fumarate was added during the course of the experiment, the respiration was stabilized at the exact time of the addition of the fumarate. It was only by comparison with a control sample, in which the rate of respiration continually diminished, that fumarate "increased" respiration.

That the respiration catalysed or stabilized by fumarate is normal to the cell was suggested by the fact that such respiration is inhibited by cyanide. It is highly probable that the systems catalysed by fumarate are in some way linked up with the cytochrome system.

Another interesting observation, incidental to our work on fumarate respiration, was made with boiled extracts of muscle or yeast. Such extracts markedly increased respiration, increases of 300% having been observed. Small amounts of extract furthermore increased the sensitivity of the tissue to fumarate, and with certain tissues which did not respond to fumarate, especially in phosphate buffer, addition of extract plus fumarate resulted in normal respiration. The extracts were not only active when added to ordinary minced muscle, but they increased the respiration of muscle which had been ground with sand until the cells were completely destroyed. Such ground muscle showed a very low oxygen uptake, but when supplemented with extract plus fumarate, normal respiration was observed. Neither addition of fumarate nor of extract alone produced normal respiration. As far as we are aware, this is the first time that the respiration of ground muscle has been increased to the normal value of unground muscle.

SUMMARY

A detailed manometric study of the effect of fumarate on the respiration of muscle was made. The following observations suggest that fumarate acts catalytically.

1—The oxygen uptake of tissues is increased by very small amounts of fumarate, and the increase is much greater than can be accounted for by the oxidation of the fumarate itself.

2—Small amounts of added fumarate are detectable after prolonged contact with respiring tissue.

3—The increased respiration is normal, that is, the respiratory quotient is not materially changed by the addition of small amounts of fumarate.

4—Substances which yield fumarate on contact with tissue—succinate, malate, oxalacetate—show a similar action to fumarate itself.

Ringer-phosphate solution has been found to be a more satisfactory medium than plain phosphate buffer to demonstrate the activity of fumarate and related compounds.

Heated muscle extract markedly increased the respiration of muscle. The oxygen uptake of ground or unground muscle in plain phosphate or in Ringer-phosphate buffer could be increased by the addition of a small amount of extract plus fumarate.

We are indebted to Professor D. Keilin for the facilities afforded to us and for his constant interest and valuable advice.

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A Quantitative Study of the Total Fat Content of Developing Salmon Eggs

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INTRODUCTION

An investigation into the problem of foodstuff metabolism involves chemical studies of the embryo and the supply of food at various stages of development. Naturally those eggs which are easy to rear and which contain large food stores in the form of yolk provide the most convenient material for an approach to the problem. Knowing what foods the egg contains at the beginning of development, the absorption processes can be studied by separate analyses of embryo and yolk throughout development. By this method, Needham (1927*a, b*) and other workers have been able to follow the protein, fat, and carbohydrate metabolism of the chick from the third day to the end of incubation. The results indicate a succession of energy sources in the order: carbohydrate, protein, fat. This phenomenon is probably of wide significance, but generalizations upon the matter are unsafe until it is known whether or not a similar situation exists in other embryos.

The salmon was chosen because its eggs are larger than those of other common teleosts and can be maintained in the laboratory with very little attention. It seemed reasonable to expect that if there were fundamental laws governing the fat metabolism of vertebrate embryos these would be disclosed by the comparison of members of two such widely separated groups as the birds and the bony fishes. In planning the investigation it was desirable to secure data similar to those already existing for the chick in the hope that direct comparisons might be facilitated. For this reason we have estimated throughout development: (1) the total fat content of the embryonic system (embryos plus yolk); (2) the total fat content of the yolks only; (3) the total fat content of the embryos only; (4) the weights of separated embryos.

From these measurements there have been estimated: (1) the fat burnt up; (2) the fat stored; and (3) the fat absorbed. These quantities bear simple relationships to the direct measurements of total fat. The fat burnt up is equivalent to the fat that is lost during development, and is

obtained from the study of the total fat of the system (embryos plus yolks). The fat stored is another term for the fat contained in the embryo detached from the yolk sac. The sum of the fat burnt up and the fat stored yields the third quantity, the fat absorbed.

METHODS

Eggs of the Atlantic salmon were obtained a few hours after fertilization from the Dominion Government Fish Hatchery at Bedford, N.S., and removed to the laboratory where they were reared in a small hatchery. Samples of 100 individuals were taken periodically for fat extraction, whole eggs and larvae as well as separated embryos and yolks, and also one sample of shells being used. Unfertilized eggs were also available for comparison. Wet weights were always taken when the material consisted of whole eggs or larvae. Dry weights of embryos only were also determined whenever possible, but time did not permit gathering a complete new set of embryonic weights. The period of observation terminated when the yolk sac had practically disappeared, about 105 days after fertilization at a temperature of 6 to 12° C. The fats were extracted by the wet alcohol-ether method described by Fowweather (1926) for lipoids in stools. The alcohol-ether extract was dried in a partial vacuum at room temperature and re-extracted with petroleum ether. It was then made up to known volume and stored in cork-stoppered bottles until required for analysis, the stoppers being sealed by several coats of silica (water glass) to prevent evaporation.

The total fat was estimated by a micro-method devised by Backlin in 1930, described by Peters and Van Slyke in 1932, and slightly modified by Van Slyke, Page, and Kirk (1933). This method provides accurate measurements of quantities of fat of the order of 0.5 milligram, and thus it is of particular value for work on early embryos where the quantities available are small. The method in principle is as follows. A sample containing about 1 mg. of fat is placed in a special test tube attached to a Van Slyke volumetric or manometric gas apparatus. Sulphuric-dichromate is added and the mixture boiled until all the fat has been converted into carbon dioxide. The CO_2 is carried over and reacts with dilute NaOH in the chamber of the Van Slyke apparatus, being subsequently liberated by the addition of an excess of lactic acid and measured as a gas. The errors resulting from extractions and fat determinations are probably within 2%. Duplicate determinations by the wet-combustion method have agreed consistently, and the wet-extraction method has proved very reliable on other material.

The calculation of the quantity of fat represented by a volume of carbon dioxide is effected in the following manner. Cubic-centimetres of carbon dioxide are first converted into milligrams of carbon by multiplying by the factor $\frac{(1.98 \times 12)}{44} = 0.540$. The conversion of milligrams of carbon

into milligrams of fat is based upon the assumption that salmon fats, like most animal fats investigated, is 76.7% carbon (Kirk, 1934). This makes a final factor of $\frac{(0.540 \times 100)}{76.7} = 0.705$. The true factor will only be

known when the fatty constituents are worked out. However, preliminary results indicate that this correction will be less than 10%, and that the total fat values as calculated are satisfactory for the present phase of the work, particularly in view of the fact that our conclusions are based on changes during development and not on absolute values at any one point. The results appear in figs. 1 to 6. In all cases the fat content and other derived quantities are measured and plotted against the age of the embryos as the independent variable. Ages have been expressed as the number of days before or after the central date of hatching. We have found this system, introduced by Allen (1932) in this laboratory, less susceptible to error than the usual one of stating the number of days after fertilization. For example, out of a million eggs in the hatchery, five thousand are taken to the University for experiment on the day of fertilization. Two months later another five thousand are brought in. In the interval the hatchery water was about 1° C. and the tap water about 6° C. The first batch may have hatched—the second batch will not show pigmented eyes. To compare the two with reference to the number of days after fertilization is meaningless. On the other hand, by the time the second batch hatches, it will be in substantially the same condition as the first. The embryos may be a trifle larger, but this is corrected for and is in any case negligible. The hatching date thus provides the best point about which to pivot the early history. Even when one selects the central hatching date as zero, the independent variable, age, is not a constant because it is related to the temperature which could not be completely controlled with the available facilities. As the temperature was a degree or two higher at the end of the experiments owing to the onset of spring weather, there has probably been a telescoping effect in this region. (It ought to be noted that the same sort of increase in temperature towards the close of the yolk sac period is met by the egg in nature.) We have made no attempt to correct for this.

Quantities of fat and dry weights have been expressed in milligrams per individual. This absolute set of values is more serviceable than a

percentage system because extraneous phenomena such as water losses or gains, or gradual disappearance of solid as development proceeds, do not make apparent changes in fat concentration when none really exists. The variation in the size of the egg batches (depending apparently upon the year, the size of the parent fish, and its place of origin) introduced an important correction. A standard hypothetical unfertilized egg was assumed with a wet weight of 130 mg., because this appeared to be a reasonable weight for eggs in early stages. Thus for eggs obtained in the winter of 1934-35 whose average wet weight for the unfertilized and early stages was 94.5 mg., the results were multiplied by the factor $\frac{130}{94.5} = 1.376$. Similarly in 1935-36, when the average wet weight was 163 mg., the factor was $\frac{130}{163} = 0.798$. The dry weights of embryos were also adjusted on this basis and the factor was $\frac{130}{154.5} = 0.841$ for the embryonic weights obtained by Allen.

THE TOTAL FAT OF THE EMBRYONIC SYSTEM (EMBRYOS PLUS YOLKS)

Fig. 1 represents the changes in the total fat content of whole eggs and larvae throughout development. 100 eggs start off with approximately 925 mg. of fat and the larvae end up with 350 mg. at the close of the yolk period. Expressed as percentages, these values indicate that at the beginning 7.1% of the wet weight and 22.6% of the dry weight of the egg is fat. By the end of the observations these fractions have decreased to 3.2% and 10.8% respectively. Moreover, 61.9% of the fat originally present has been lost, *i.e.*, burnt up to provide energy for the metabolic processes.

The results for the intervening period show that until the fourth week after hatching no major variations in fat content occur. At the 25th day after hatching, however, fat begins to disappear rapidly and this process continues unabated for about a week. When the observations ceased, fat was still being lost but less rapidly than before. It has already been mentioned that the fat lost during development represents the fat burnt up. Fig. 1 shows the quantities of fat burnt up as development proceeds. These quantities were determined by subtracting the total fat figures from the average figure for the early period, during which fat was not being burnt up (924 mg. per 100).

With regard to the pre-hatching period, we have, unfortunately, observations on only two batches of unfertilized eggs. We are unable to

decide between them and we hesitate to average them, so two possibilities of the beginning of the total fat curve are shown. Whether there be a loss of fat in the earliest stages or not, it is reasonably certain that there is a fat synthesis from -20 days almost up to hatching. As mentioned below, this is a confirmation of previous results. The synthesis of fat in early embryology has now been demonstrated for so many forms (though the mechanism is known for none) that it may be taken as a general phenomenon. For references *see* Needham (1931). We return to this point in the discussion.

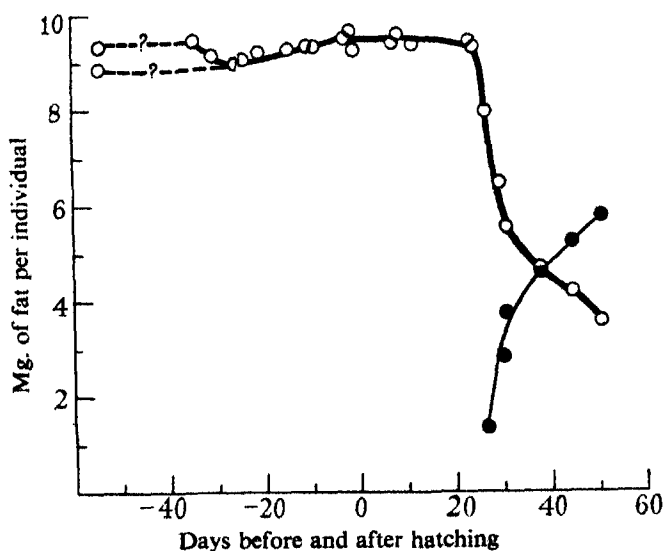


FIG. 1.—The total fat in the egg or larval system (embryo plus yolk) and the combustion of fat by the system. ○ Total fat in egg or larval system (embryo plus yolk); ● fat burnt up.

The total fat of the salmon embryonic system has been investigated twice previously. Many years ago Miescher (1866) reported a loss of 60% of the fat between fertilization and the disappearance of the yolk sac. His two solitary analyses are entirely confirmed by our figures. The other study was in a previous paper by one of us (Hayes, 1930, *b*). If fig. 3 of that paper is compared with fig. 1 of this, it will be seen that the older results :

- (1) begin at a period corresponding to about -20 days, and show the same slight fat increase previous to hatching;
- (2) show a marked fluctuation at the time of hatching which has not appeared in the newer analyses;

- (3) exhibit the same drop 4 weeks after hatching as the current results, although the observations were not carried quite to the end of the yolk sac period;
- (4) are lower than the new by about one-third, possibly to be accounted for in part by difference in technique (Soxhlet extraction with carbon tetrachloride and subsequent weighing in the older work; choice of a factor as mentioned above in the newer).

The older and newer curves are essentially similar, provided that the points which fall within the hatching period are neglected. We believe that the marked differences at hatching time are to be sought in the incubation temperatures. Practical hatchery men know that egg mortality near hatching is accelerated at high temperatures, and it is possible that 10° was close to the lethal temperature at this critical period. Further work on the point is necessary before a complete explanation can be given.

One fat extract of salmon egg shells or capsules was prepared and a value of 1.87 mg. per 100 shells was obtained, *i.e.*, less than 1% of the wet weight and about 3% of the dry weight of the shell. This determination was made at the time of hatching. Since this quantity is of the order of 0.2% of the total fat in the egg, it will be neglected in subsequent discussion.

THE TOTAL FAT OF SEPARATED YOLK SACS

All the relationships of fat which we discuss could be derived from analyses of any two of the following three: whole larva, embryo only, yolk only. Our estimates are based on the first two; but they were checked at ten points by analyses of 100 yolk sacs separated from the embryo by tearing off the outer yolk membranes. The yolk is kept intact by the inner membranes consisting of endoderm and splanchnic mesoderm.

The results of the total fat determinations for the yolk sacs parallel the fat in the system as a whole, but the yolk sac figures fall off somewhat more rapidly as the end of development is reached. This is due to the increasing amount of fat stored in the embryo. It may be noted that the fat lost by the yolk, because it is equal to the fat burnt up plus the fat stored, is a measure of the fat absorbed, according to the terminology which is in use in this paper. The yolk sac analyses are not given since they confirm entirely the other results.

FAT OF EMBRYOS ONLY (FAT STORED)

Beginning about three weeks before hatching, the fat content of the salmon embryo was measured at average intervals of eight days. The results are given in fig. 2. It will be remembered that these values also express the amount of fat stored as previously defined.

The absolute values in fig. 2 indicate that the storage of fat is interrupted at the time of hatching. Whereas 100 embryos contain 26.4 mg. of fat 2 days before hatching, on the eighth day after hatching 100 embryos contain only 21.8 mg., a drop of almost 20%. As this is well beyond the experimental error, the suggestion of a hatching hold-up in fat storage cannot be explained away as an error in technique. A superficial examination of the curve of absolute values in fig. 2 would suggest that only two

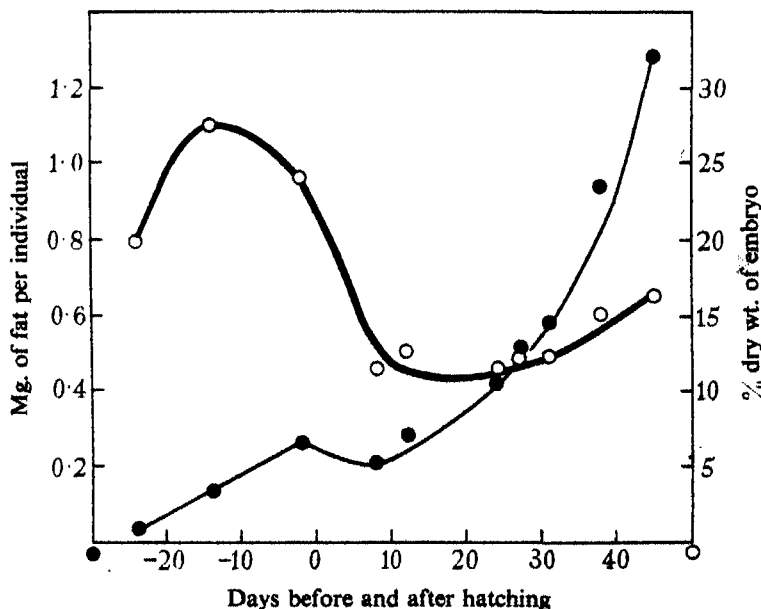


FIG. 2.—The total fat content of salmon embryos, removed from the yolk sac, expressed in absolute values ●; and as a percentage of dry weight ○.

points need to be eliminated if the apparent drop in fat content at hatching is to disappear. There are, however, several pieces of evidence to suggest that the curve as drawn is correct. First of all we are indebted to Dr. C. H. Goulden, Statistician at the Dominion Rust Research Laboratory, Winnipeg, for the following statement.

“Application of analysis of variance (Fisher, 1932) shows that the slope of the first part of the curve of absolute values in fig. 2 is significantly

different from that of the second part, and that the break in the line (appearing in the curve as a dip at the 8th day) is also significant. Data are not sufficient to show whether the dip is to be accepted as such or as a failure to continue the upward slope of the graph after the first three days."

Secondly, evidence from the literature of hatching phenomena shows that a comparable stoppage of other processes occurs. For instance, the growth rate of trout shows a decided falling off during the period of hatching (Scheminsky, 1929). Moreover, Saunderson (1935) in this laboratory has shown that the rate of cartilage formation in the salmon is unmistakably diminished while hatching is in progress. Finally, a consideration of the water-salt relationships has led to the conclusion (Hayes, 1930, *b*) that "at hatching the embryo is suffering from malnutrition, both embryo and yolk are lacking in water, but both have maintained their salt content. In the larval condition the embryo immediately begins to recover". Thus in the final analysis, we are justified in assuming that stored fat is used by the embryo at the time of hatching.

It is interesting to study the changes in the percentage of fat in the embryo. As a preliminary, figures must be provided for the dry weights of embryos at various stages, and these have been obtained from a curve constructed from the dry embryonic weights of Allen (1932), with additional dry weights determined by the writers, and corrected, as explained before, to the standard 130 mg. egg. Unfortunately, no wet weights are available for the salmon, and the dry weights are not so satisfactory as if one investigator had made the whole set. However, until more complete figures are available, they must suffice for the calculations.

Returning to the embryonic fat, fig. 2 shows that, expressed as a percentage of the dry weight, it reaches a maximum about two weeks before hatching and that the minimum exists at the close of the hatching period. As observations ended this quantity was rising rapidly. Until more embryonic weights are obtained the pre-hatching maximum is somewhat doubtful, but the hatching minimum and the final rise appear to be reliable, the latter especially, as it has also been observed in the chick by Murray (1926). (Comparison with fig. 4 shows that there is no simultaneity between the maxima of combustion and storage.)

THE FAT ABSORBED

The sum of two quantities already determined (fat burnt up plus fat stored) has been defined as the *fat absorbed* by the embryo from the yolk

sac. This quantity is important because it measures the movement of fat into the embryo. If the amounts of fat absorbed throughout development are plotted against age, fig. 3 is the result. It is simply the addition curve of fig. 1 ("burnt up") and fig. 2 ("absolute"). It will be noted that all the fat absorbed up to the fourth week after hatching is stored in

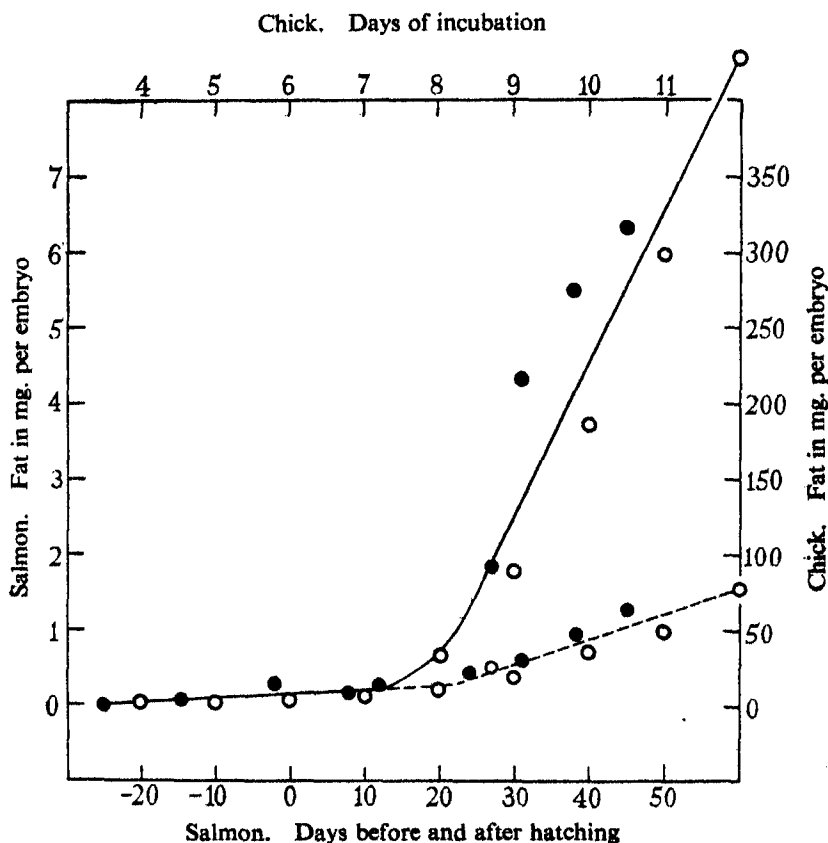


FIG. 3.—A comparison of storage and absorption of fat from the yolk sac in salmon and chick embryos. Absorption equals storage plus combustion. Figures for the chick are from Needham (1927a, Table VIII, fat stored being col. 3 and fat absorbed being 5600 plus col. 3 minus col. 2). ● Salmon; ○ chick; — absorption; --- storage.

the embryo. However, when combustion starts, the two curves diverge and at the 45th day the amount of fat absorbed is approximately five times the fat stored.

Fig. 3 acquires significance when it has superimposed on it a curve plotted from Needham's data for the fat absorbed by the chick during development. Fat is not burnt up in the chick until the seventh day of

incubation. Up to this point the storage and absorption curves are identical, but then they diverge very rapidly in a manner not unlike the same curves for the salmon. Not only are the general trends the same for both embryos, but the rates of divergence of the absorption and storage curves are approximately the same. Further implications of this situation will be discussed later.

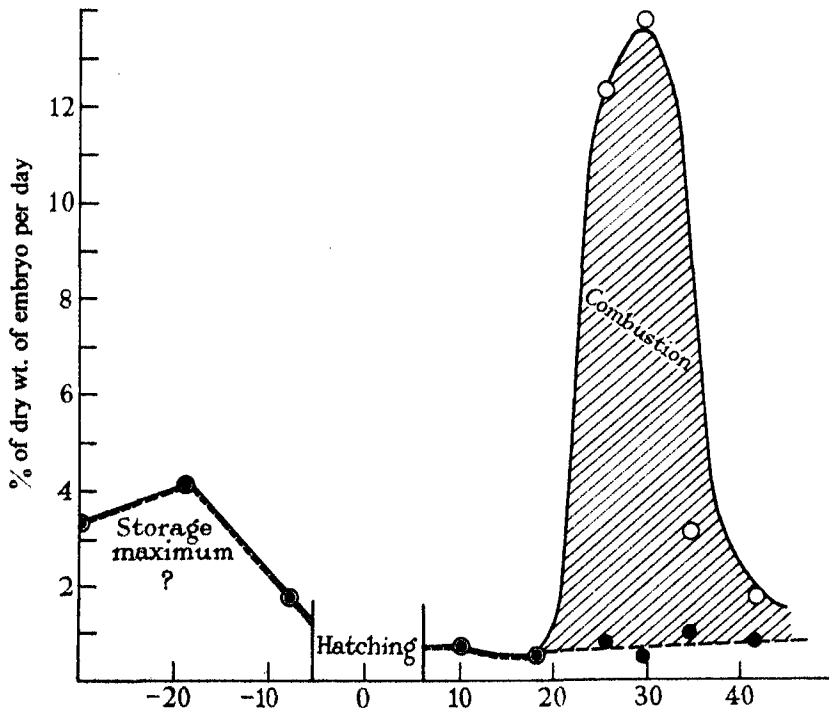


FIG. 4—Rates of storage and absorption of fat in salmon development (absorption equals storage plus combustion). —○— rate of absorption; —●— rate of storage.

RATES OF COMBUSTION, STORAGE, AND ABSORPTION

When daily increments of fat burnt up, stored, and absorbed have been calculated they may be related to the embryonic weights so as to give the rates of these respective processes. That is, we can know what percentage of its dry weight the embryo stores, burns, or absorbs every day. The results of such a calculation are plotted in fig. 4. It will be observed that these are mean values and the ages are taken as mean ages.

While the pre-hatching section of fig. 4 cannot be regarded as conclusive until a better set of weights is available, the post-hatching absorp-

tion maximum of 14% is doubtless a fact. The apparent lack of a relationship between rates of combustion and storage is too well emphasized in the figure to require further mention here, but its significance will appear later.

Fig. 5 illustrates a calculation made from Needham's (1927, *a*) data. A glance shows that it is very like our fig. 4, and that combustion and absorption of fat are pre-eminent at the same time. Now, as Needham

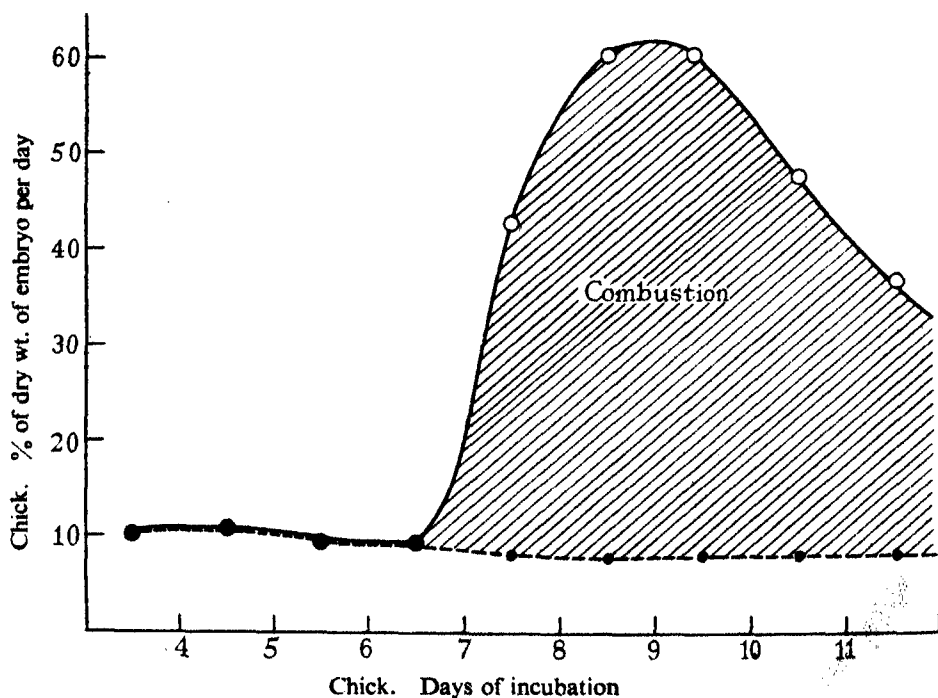


FIG. 5—In Needham (1927, *a*) Table IX, cols. 2 and 4, are figures for fat storage and absorption by chick embryos during development. These have been recalculated as percentages of the embryonic dry weight using Murray's (1926) weight estimations together with those of Needham (1927*a*). —○— rate of absorption; —●— rate of storage.

points out, the fat loss as determined by his chemical analyses is considerably in excess of that lost as determined from the carbon dioxide output. According to the respiratory quotients, there should be no chronological relation whatever between combustion and absorption, yet as our analyses show for the salmon and Needham's for the chick, the two processes occur at the same time. Needham prefers the evidence of CO_2 output to the analytical results, but in view of the confirmatory analyses from the salmon, as shown in fig. 4, it would appear that absorption

and combustion maxima for fat are simultaneous processes in both forms.

Indeed, when one considers that in the chick as well as in the salmon the accepted relationship is:

$$\text{combustion plus storage} = \text{absorption},$$

it is difficult to see how a peak in combustion could accompany a trough in absorption unless a radical diminution in the storage rate took place at the same time, and the storage rate represented a major proportion of the absorption. Neither of these requirements is met.

ABSORPTION INTENSITY OF FAT IN THE SALMON

Needham has introduced a quantity, absorption intensity, into the study of the chick which provides a basis for the comparison of metabolic processes in different embryos. If fat absorbed per day is expressed as a percentage of the fat remaining to be absorbed, a set of values is obtained in which the maximum is on the 29th day.

If these quantities are related to embryonic weights so that a calculation is made for the percentage of the remaining fat that 100 gm. of embryo absorbs per day, we have a measure of the absorption intensity. Fig. 6 shows the variations in this quantity and the curve is known as the "absorption curve". The maximum on this curve occurs at the point where the most rapid combustion of fat observed in fig. 1 is going on. It is impossible to follow the absorption of fat through the hatching period on account of the fall in stored fat. It is probable that the absorption of fat at this time is at least partly suspended.

Needham's absorption curve of fat in the chick has been superimposed on fig. 6, and it is clear that there are many points of agreement. An initial period of low intensity is followed by a sudden rise to a maximum of 1300% at 9 days. In the salmon the rise takes place at 2260% at 29 days after hatching and the question naturally arises whether the salmon at this age is the equivalent of the 9-day chick as regards the extent of its development. The agreement here, as in the previous figures, can hardly be a chance one, and we are at least justified in saying that the succession of phenomena in the two animals is the same. The implications of this are considered in the next section.

If one were to draw the left halves of the curves in fig. 6 through all the points (rather than straight lines) it would make, for both salmon and chick, corresponding minor peaks early in development. Even though the peaks are in each case small and are dependent on one point only,

may it not be that there is an absorption intensity maximum earlier in development than has been suspected ?

DISCUSSION

An examination of figs. 3 to 6 suggests that, on the basis of fat metabolism, the period of salmon development up to the absorption of the yolk sac

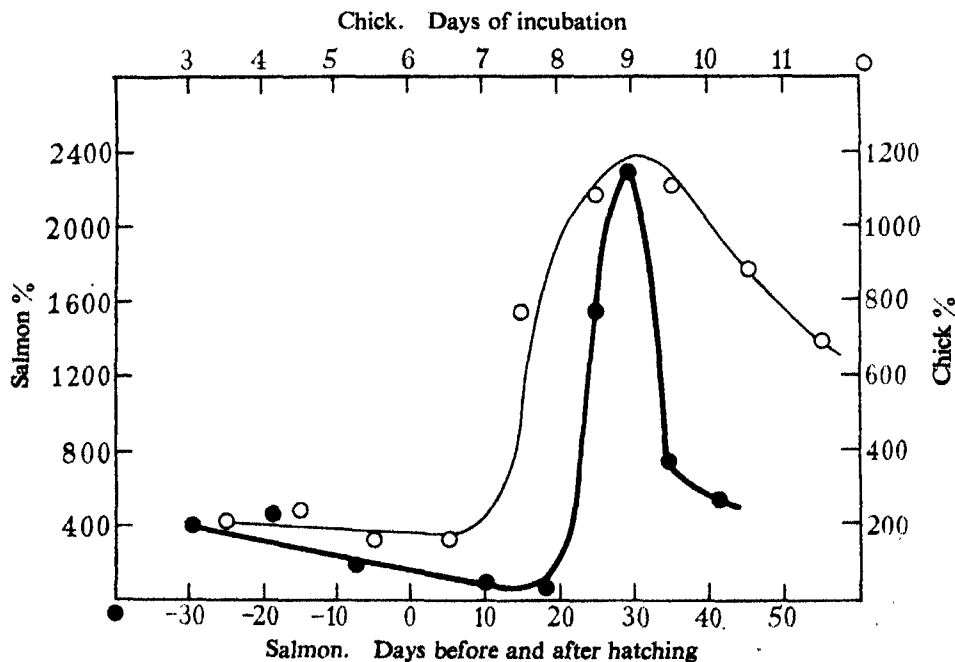


FIG. 6—A comparison of the intensity of fat absorption in salmon and chick development. Figures for the chick are from Needham (1927, *a*, Table IX, col. 10). The points represent the amount of fat which would be absorbed in one day by 100 gm. of dried embryo, expressed as a percentage of what remains in the yolk sac to be absorbed on that day. ○ chick; ● salmon.

may be compared to the first 12 days of chick development. It should be emphasized that the same technique of superimposition has been followed throughout, namely to place the sixth day of chick development over the zero point of salmon development (hatching), and to let one day in the chick correspond to ten in the salmon.

The surprising similarity of the curves led us to look to morphology for possible confirmation. Morphological comparisons are made difficult by the fact that no complete chronology exists for the development of either the salmon or the chick. However, by using data for the

early embryology of both forms, as found by reference to Lillie (1919) and Ziegler (1882), the following computations have been made. Salmon ages are reckoned as days after fertilization, rather than as elsewhere in this paper.

Embryonic period of the salmon at 10°, 120 days (Allen, 1932; Hayes, 1930, *a*).

Age of salmon at closure of medullary folds, 10 days (Ziegler, 1882).

Age of chick at closure of medullary folds, 1 day (Lillie, 1919).

Thus 120 days in salmon = $\frac{120 \times 1}{10} = 12$ days in chick.

Age of salmon at appearance of pronephros, 16 days (Ziegler, 1882).

Age of chick at appearance of pronephros, 1½ days (Lillie, 1919).

Thus 120 days in salmon = $\frac{120 \times 3}{2 \times 16} = 11\frac{1}{4}$ days in chick.

Age of salmon at appearance of liver, 20 days (Ziegler, 1882).

Age of chick at appearance of liver, 2 days (Lillie, 1919).

Thus 120 days in salmon = $\frac{120 \times 2}{20} = 12$ days in chick.

Age of salmon at hatching, 50 days.

Age of chick at completion of vitelline membrane rupture, 5 days (Lillie, 1919).

Thus 120 days in salmon = $\frac{120 \times 5}{50} = 12$ days in chick.

It will be seen that the data of organogenesis permit calculations of this kind to be made with very good agreement. Furthermore, if such morphological equations are permissible, the inference which was drawn from the comparison of the fat metabolism of the two embryos is correct; namely, that the salmon only carries the story of development as far as the twelve-day old chick, or in other words at our temperatures, 10 days in the salmon = 1 day in the chick.

There is a second factor which enters into a comparison of the fat metabolism of the salmon and the chick. It arises from the fact that in the chick, the fat combustion comprises a much larger proportion (91%) of the total material burnt up than in the salmon (30%—Miescher, 1866). This effect, operating in conjunction with the longer period of dependence upon the yolk, would have a flattening effect upon the absorption curve for the chick, because the values depend upon the amount of fat remaining to be absorbed. This explains the greater magnitude of the peak in the absorption curve of the salmon. Otherwise one would expect a peak of greater magnitude in the chick where the metabolic rate is much higher.

Assuming that our age comparisons of the salmon and chick are correct, it is instructive to see where the chick succession of energy maxima would occur in the salmon. (For carbohydrate and protein there are, of course, no salmon figures available.)

Chick days	Chick peaks of absorption	Days of salmon which correspond
4	Carbohydrate	-20
4	Protein	-20
9	Fat	30
15	Protein	Post yolk sac
19	Fat	Post yolk sac

It will be observed that the last two absorption maxima in the chick have no correspondence to the period during which the salmon lives on its yolk. It is obviously unsafe to assume that the succession of energy sources known to occur in the chick (where hatching occurs in an advanced developmental stage) can be transferred unchanged to other forms.

It is desirable to enquire whether respiratory quotients, as studied in other teleosts, can be made to throw any light on our fat analyses. Amberson and Armstrong (1933) made a very careful study of respiration in *Fundulus* up to the time of hatching (12 days). Probably hatching in *Fundulus* and the salmon are morphologically comparable; at any rate the time of establishment of the circulation in the two forms relative to the time of hatching is the same. Their curve for the R.Q. is U-shaped, dropping from 0.9 on the first day to 0.69 on the eighth, and then rising gradually to 0.74 at the close of the observations. The middle figure, 0.69 (which is the average of seven observations) is significant because it is too low to have been produced by combustion even of pure fat. It could, however, easily be accounted for by a transformation of fat into carbohydrate. If the total fat curve in our fig. 1 is now examined it will be seen that in the pre-hatching phase there could be a drop up to about -20 days followed by a rise to the original value by hatching time. The fat synthesized shortly before hatching is added to the yolk sac and not to the embryo. The lowest value in our pre-hatching fat curve corresponds to Amberson and Armstrong's lowest R.Q. in that both occur about two-thirds of the way to hatching. In view of our failure to detect any significant combustion of fat until some weeks after hatching, we are forced to conclude that if *Fundulus* and the salmon are comparable, the low R.Q. is due to a transformation of fat into carbohydrate. In the absence of chemical evidence, we suggest carbohydrate for two reasons. First because this would produce a lower R.Q. than would protein

synthesis; and second because in the chick according to Needham (1927, *b*) "some 40 mg. of carbohydrate rather suddenly appear from somewhere, and there is at the same time a quantity of missing fat of the same order". A synthesis of fat, such as we observed in the stages shortly before hatching, ought to result in a rise in the R.Q., and that is what happens in Amberson and Armstrong's curve. Their value for the R.Q. just before hatching was 0.74, which is too high to represent pure fat combustion.

We are not sure what is being combusted in the pre-hatching phase, and we suspect that the R.Q., complicated as it is by the curious behaviour of the fat, is not to be taken at its face value.

The work of Schlenk (1933) on the respiration of the trout confirms Amberson and Armstrong's results for the period before hatching in that (*a*) the lowest R.Q. found occurs about two-thirds of the way to hatching, and (*b*) having a value of 0.58, it is too low to be accounted for even by pure fat combustion. Schlenk continued his observations after hatching up to a period which would correspond to about 22 days in our salmon nomenclature, which, as a glance at fig. 1 will show, is just before fat begins to be removed in large quantities. Unfortunately, therefore, Schlenk's results cannot be used to check our final fat drop. In the period which corresponds to 8-22 days in the salmon, the R.Q. is probably 0.78, suggesting pure protein combustion, while for the fortnight which has hatching as its centre, 0.65 is a probable figure.

It has been observed that although lipoid materials are taken from the yolk and stored in the body of the embryo for tissue-building purposes from the beginning of development, appreciable quantities of fat are burned only in the last four weeks. There could be two explanations of this phenomenon; either the embryo lacks the ability to burn fat, or it lacks the ability to absorb it in large quantities. If the observed loss of fat from the embryo at the time of hatching, presumably due to combustion, is a fact, the first explanation is impossible. It would then appear that large quantities of fat cannot be absorbed under circumstances that are adequate for storage requirements. Either a new factor in fat metabolism or perhaps a new mechanism for absorption appears at the point where combustion begins. Needham has met the same problem in the chick and he explains the changes in absorption intensity by the hypothesis that the blastodermal blood vessels undergo changes in permeability to fat. Now in the chick a mechanism not depending upon the vitelline circulation is inconceivable. In the salmon, however, where the yolk sac never loses its direct association with the mid-gut by the development of a yolk stalk, absorption directly into the embryo's digestive system is at all events a possibility. Moreover, the manner in which the large oil

globule takes up its position immediately beneath the liver and the mid-gut might well be a significant phenomenon. It is noteworthy that bile pigment first appears in the gut about three weeks before hatching which, as fig. 4 shows, probably marks the storage maximum.

It is a pleasure to acknowledge the friendly interest and cooperation of our colleagues, Professors D. Pelluet, E. G. Young, and D. Mainland.

SUMMARY

The total fat in whole eggs and larvae, and in the embryos and yolks of the salmon, has been estimated quantitatively throughout development. The major period of fat combustion (probably the only one) occurs during the last four weeks of the yolk sac period. The total fat of the embryo alone rises rapidly at first, is reduced at hatching, then recovers and increases steadily until the end of embryonic life. The total fat of the yolk sac rises before hatching and later falls, slowly at first; but the rate of diminution is greatly accelerated when combustion of fat begins.

The curves for total fat absorbed and stored by the salmon have been compared with similar curves for the chick, drawn to a scale such that the end of the yolk sac period in the salmon corresponds to the 12th day of incubation in the chick, and similarity has been noted in essential features. The assumption that the ages correspond at the superimposed points has been supported by morphological comparisons.

Fat-absorption curves to show intensity also coincide in the salmon and the chick if the 12th day of chick development is assumed to mark the end of embryonic life in the salmon. The peak of absorption is on the 29th day after hatching, corresponding to the 9th-day peak in the chick.

There appears to be a relation of simultaneity between the absorption and combustion of fat.

The rate of fat storage by the embryo is quite unaffected by the sudden rise in the absorption rate.

It is inferred from the results that the sequence of energy sources in ontogeny (carbohydrate—protein—fat) known to occur in the chick is probably also true for the salmon.

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The Dependence of Pupil Size upon External Light Stimulus under Static and Variable Conditions

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This investigation forms part of a general investigation of the variation of visual sensitivity with time in consequence of a change in the external light stimulus. Part of the variation in sensitivity of the eye as a whole is due to change in pupil size, and this pupil size factor may conveniently be considered separately, and afterwards combined with the variation of the intrinsic sensitivity of the retina of the eye; this is measured with fixed pupil size (artificial pupil). The investigation of the pupil size factor falls naturally into two parts. One must first know what is the pupil size for any static condition in the subject's field of view, that is, for any primary conditioning stimulus. Then follows the determination of the variation of pupil size with time after the change in the external light stimulus. The case investigated here is the change from a high to zero stimulus, consideration being given also to the dependence of the variation on the initial stimulus level.

1—THE DEPENDENCE OF PUPIL SIZE UPON THE LIGHT STIMULUS APPLIED TO THE EYE UNDER STATIC CONDITIONS

Some aspects of this part of the subject have been investigated by a number of authors. Couvreur (1924), Lythgoe (1936), Reeves (1920), and Schroeder (1927) give results showing the dependence of pupil size upon the general background brightness, approximately uniform over a large part of the field of view. Holladay (1926), in addition, gives results for a single glare source in presence of a uniform background, the glare source being at various angular separations from the fixation point. The results of Schroeder may be ruled out of consideration in the present connexion, as his method automatically measures the effective, not the real, pupil size, allowing for the pupil efficiency effect (Stiles and Crawford, 1933). Couvreur and Holladay used a double-pinhole pupillometer, which is an undesirable method as the subject has to concentrate on making the measurement and is not under the sole influence of the light stimulus to be tested. Lythgoe and Reeves photographed the

pupil by flashlight exposure. This is the only really satisfactory method of universal application and is the one chosen for the present work. For most of this, infra-red photography has been employed, with Ilford infra-red-sensitive ciné film and Ilford infra-red lamp glass as filter in front of the illuminant. The latter was a 500-c.p. pointolite lamp focussed by a condenser on to the eye to be photographed. A certain amount of red light is visible through the Ilford filter, but this was found not to be disturbing with a time of exposure of $\frac{1}{20}$ second or less. The pupil diameters given in what follows are those measured on the photographs directly, without making any allowance for corneal refraction, that is, the apparent pupil diameters as seen from outside the eye, not the actual diameters.

Pupil size is influenced by the brightness of the general field of view, by its size and by glare sources in it, both in respect of their intensities and their positions relative to the direction of fixation. It would be desirable to be able to represent all these effects in a single formula, or at least in a consistent family of curves. The results obtained do not so far render this possible, and the best one can do is to investigate the more complex conditions individually.

1 (i)—*The Variation of Pupil Diameter with Angle between Glare Source and Fixation Point*—The glare source used was a 100-watt, 100-volt projector lamp mounted on a pivoted arm so as to swing in a horizontal plane about an axis passing through the point midway between the subject's eyes. The distance from the glare source to the eyes was 1.8 metres. The subject's head was fixed in a definite position by biting on a mouth-piece of sealing wax. The glare angles were taken in random order, the subject being given $1\frac{1}{2}$ minutes' adaptation at each angle before the photographs were taken. The glare intensities were taken in order of increasing intensity. Ten subjects were chosen at random for the measurements. A large degree of individual variation is shown, also great irregularity in the individual curves. Such irregularity would appear to be much greater with a glare source in an otherwise dark field than with a uniformly bright field centred at the fovea.

The results are given in Table I. The experimental points can be fitted tolerably well with curves of simple exponential type:

$$\left. \begin{array}{l} \text{For } E = 0.00347 \text{ ft. c., } D = 0.78 \quad (1 - e^{-0.075\theta}) + 5.65 \\ \text{For } E = 0.102 \text{ ft. c., } \quad D = 1.29 \quad (1 - e^{-0.075\theta}) + 4.45 \\ \text{For } E = 4.37 \text{ ft. c., } \quad D = 1.73 \quad (1 - e^{-0.075\theta}) + 3.40 \end{array} \right\}. \quad (1)$$

E is the illumination at the subject's eyes from the glare source, D is the pupil diameter, θ is the angle in degrees subtended at the eye between the glare source and the fixation point.

TABLE I.—PUPIL DIAMETER (MM.) FOR SUBJECT:

Glare angle (°)	H. R. S.	B. H. C.	F. J. C. B.	F. M. H.	W. S. S.	C. D.	G. W. G. S.	G. M. N.	L. J. C.	H. B.	Mean
0	6.1	6.6	6.1	3.3	5.7	5.3	6.4	5.7	5.7	5.5	5.64
7	5.8	7.0	6.8	4.1	6.2	5.8	6.2	6.4	5.8	5.4	5.95
14	6.3	7.3	6.6	4.4	6.5	5.7	6.4	6.8	6.0	5.7	6.17
21	6.1	7.9	7.3	4.6	6.5	5.9	5.8	7.0	6.0	6.3	6.34
28	7.0	7.3	7.1	4.6	6.3	5.7	6.6	6.8	6.0	6.4	6.38
35	6.3	7.1	7.2	4.6	6.4	5.8	6.6	7.0	5.9	6.5	6.34
42	—	6.6	7.4	5.0	6.6	6.1	6.4	—	6.0	6.3	—
49	—	7.3	—	—	—	—	6.3	—	5.9	6.6	—
56	—	7.8	—	—	—	—	6.9	—	6.2	—	—
0	5.0	5.4	3.7	2.8	4.1	4.7	5.0	4.3	5.1	4.3	4.44
7	5.3	6.8	4.7	3.5	4.5	5.4	4.7	5.8	5.2	3.9	4.98
14	5.3	6.6	6.1	4.1	5.1	5.2	6.0	5.7	5.0	4.3	5.34
21	5.7	6.4	6.4	4.1	5.6	5.4	5.4	6.2	5.1	4.4	5.47
28	5.4	7.2	6.4	4.3	5.6	5.6	5.5	6.1	5.6	4.9	5.66
35	5.2	5.6	6.8	4.6	5.6	5.7	5.3	6.4	5.1	4.8	5.51
42	—	6.1	6.5	4.1	5.6	5.7	5.1	—	5.4	5.1	—
49	—	6.9	—	—	—	—	6.1	—	5.7	4.8	—
56	—	6.9	—	—	—	—	5.8	—	5.7	—	—
0	3.8	4.6	3.9	2.6	3.0	2.5	3.7	4.1	3.7	2.8	3.55
7	4.5	5.8	4.5	3.1	3.9	2.9	4.1	4.5	4.0	3.3	4.06
14	5.1	5.0	5.1	3.2	3.4	4.3	4.9	4.9	4.5	3.8	4.42
21	5.4	5.5	5.8	3.3	4.2	4.7	4.9	5.8	4.2	4.1	4.79
28	5.7	6.0	6.4	3.6	4.6	4.6	5.7	5.4	4.7	4.2	5.09
35	5.0	5.5	6.4	4.0	4.9	4.0	5.0	6.1	4.7	3.1	4.87
42	—	5.6	6.8	3.5	5.2	3.4	5.8	—	4.9	4.7	—
49	—	6.3	—	—	—	—	5.6	—	5.0	4.4	—
56	—	6.0	—	—	—	—	5.3	—	4.9	—	—

m = 0.00347 ft. c.

m = 0.102 ft. c.

m = 4.37 ft. c.

m = 4.37 ft. c.

Values for glare illuminations other than those given can be interpolated with fair accuracy, but not extrapolated, since the curve of pupil size against glare intensity flattens considerably outside the maximum and minimum values of glare illumination used.

It is possible that some simplification of results could be obtained by expressing them in terms of equivalent background brightness, that is, the actual pupil diameters would be replaced by the background brightnesses which give the same values of pupil diameter when the eyes are exposed to the uniform background only. To investigate this possibility, the variation of pupil size with background brightness was determined for each of the ten subjects of the glare angle experiment. The glare source was replaced by a white screen at a distance of 75 cm. from the subject's eyes, the diameter of the uniformly illuminated portion subtending an angle of 55°. The rest of the field of view was dark or nearly so. Accommodation was relaxed, not at the distance of the screen. The screen was illuminated by a single lamp or a projector, the brightness being varied by Wratten neutral filters. Before starting his series of photographs, each subject rested his eyes in darkness for half an hour. Photographs were then taken at a series of increasing background brightnesses, five exposures at each brightness. The above conditions for the determination of equivalent background brightness have been used throughout the rest of this paper.

The results are shown in Table II and fig. 1. A hyperbolic tangent formula (the dotted curve) has been fitted to the experimental points and represents them fairly well for purposes of calculation. It is unlikely, however, that it represents any theoretical mechanism of the effect. In fig. 1 there are also shown, for comparison, the results of Holladay, Lythgoe, and Reeves. There is enormous variation between the different sets of data, which can only be ascribed to variation between individuals. That this is not an improbable explanation may be seen from a comparison of the curves for individuals in the various investigations. The data of Lythgoe show the widest range of individual variation and all the other subjects lie within his range or nearly so. Obviously, a very large number of subjects would be required in order to obtain a truly representative mean curve.

The transformation of pupil diameters to equivalent background brightnesses follows quite simply by reading from the curve of fig. 1 the $\log_{10} B$ value corresponding to any given pupil diameter. The resulting points can be fitted by a family of curves of the exponential type

$$\beta = ae^{-b\theta} + c, \quad (11)$$

TABLE II—PUPIL DIAMETER (MM.) FOR SUBJECT:

Log ₁₀ B (C./R ²)	H.R.S.	B.H.C.	F.J.C.B.	F.M.H.	W.S.S.	C.D.	G.W.G.S.	G.M.N.	L.J.C.	H.B.	Mean
8.04	7.5	8.0	7.8	5.8	7.3	6.6	7.1	7.5	6.6	7.1	7.13
7.21	7.6	7.8	7.6	5.9	7.0	6.6	7.0	7.2	6.4	7.1	7.02
6.22	7.5	7.7	7.6	5.2	7.0	6.6	6.9	7.0	6.2	6.9	6.86
6.76	7.4	7.5	7.6	5.1	6.9	6.5	6.8	6.7	6.2	6.7	6.74
5.23	7.1	6.9	7.5	5.1	6.8	6.5	6.7	6.7	6.0	6.7	6.60
5.90	7.0	7.3	7.2	4.9	6.7	6.2	6.4	6.3	5.6	6.5	6.41
4.37	6.7	7.1	7.0	4.4	6.3	6.1	6.0	6.1	5.3	6.2	6.12
3.07	5.8	6.7	5.7	3.6	5.8	5.4	5.4	5.4	4.8	5.3	5.39
3.54	5.5	6.0	5.0	3.8	5.1	5.4	4.7	4.9	4.5	4.9	4.98
2.08	4.9	5.5	4.5	3.1	4.6	4.8	4.3	4.3	4.4	4.4	4.48
2.55	4.2	5.0	4.4	3.1	4.1	4.8	3.9	4.1	4.0	4.7	4.23
1.09	4.4	4.5	3.7	2.9	3.7	4.4	3.4	3.7	4.1	3.9	3.87
1.56	4.0	4.3	3.5	2.9	3.4	3.8	3.2	3.8	3.7	3.7	3.63
0.23	4.0	4.2	3.8	2.7	3.4	3.8	3.1	4.0	3.5	3.7	3.62
0.76	3.6	3.7	3.6	2.5	3.0	3.5	2.8	3.8	3.5	3.5	3.35
1.32	3.3	3.5	2.9	2.3	2.8	2.8	2.8	3.2	2.8	3.0	2.94

where β is the equivalent background brightness and a, b, c are constants given by the equations:

$$\log_{10} a = \log_{10} E + 1.20 \quad (\text{III})$$

$$b = 0.5 \log_{10} E + 0.269 \quad (\text{IV})$$

$$\log_{10} c = 0.5 \log_{10} E + 3.27. \quad (\text{V})$$

From a practical point of view, there appears to be no advantage in using these equivalent background formulae, which are more complicated than the direct pupil diameter formulae, and also involve the use of another

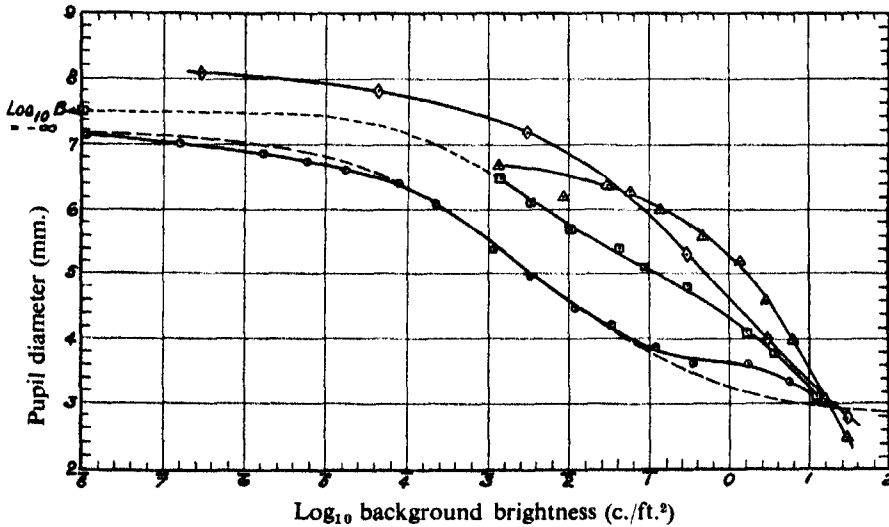


FIG. 1—Variation of pupil diameter (D) with background brightness (B). Present results, $\odot$; Holladay, $\triangle$; Lythgoe, $\square$; Reeves, $\diamond$; ---, $D = 5 - 2.2 \tanh 0.447 (\log_{10} B + 2.4)$.

formula or curve for the deduction of the actual pupil diameter. On the other hand, it is possible that there may be less variation between different subjects and groups of subjects in their equivalent background values than in their actual pupil diameters. The only comparison available is between the present results and those of Holladay. Holladay's results relate to a glare illumination at the eye of 0.107 ft. c., and can therefore be directly compared with the present results for 0.102 ft. c. eye illumination. The ratio of equivalent background brightnesses found in the two investigations varies according to glare angle between 20:1 and 60:1, no small discrepancies, but smaller than those shown in fig. 1 between the background brightnesses for given pupil diameters. These latter discrepancies range from nothing to nearly 500:1 between the present and Holladay's curves.

1 (ii)—*Variation of Pupil Diameter with Azimuth Position of Glare Source*—To ascertain the variation, if any, of pupil size with the azimuth position of a glare source, the angle between glare source and fovea and the intensity of the glare source remaining constant, apparatus was arranged so that a glare source, a 100-watt projector lamp, could be swung in a circle round the fixation point as centre. Eight equally spaced positions on the circle were taken, the glare source vertically above the fixation point being one, and the pupil size measured at each. The eight positions were taken in a different random order for each set of

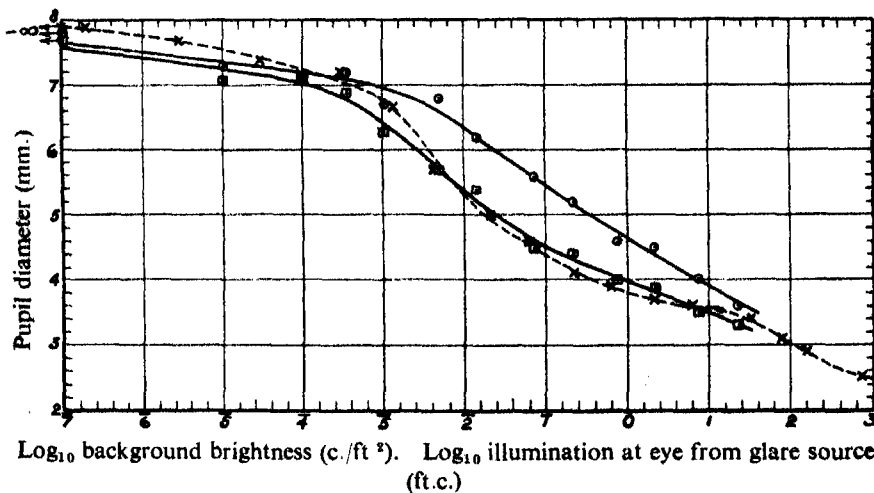


FIG. 2—Variation of pupil diameter with background brightness and glare illumination. Subject, B. H. C. Background subtending solid angle of 0.37 steradians at eye, $\times$; glare source at 5° from fixation point, $\square$; glare source at 28° from fixation point, $\odot$.

measurements. Within the limits of experimental error, no variation of glare effect occurred for the two subjects tested.

1 (iii)—*Variation of Pupil Diameter with Intensity of Glare Source at Constant Glare Angle*—The variation of pupil size with glare intensity for a given glare angle, and its relation to the variation with background brightness, may be to some extent inferred from equations (II) to (V). For zero glare angle, β is very nearly equal to a , c being small by comparison, so that equation (III) becomes, approximately,

$$\log_{10} \beta = \log_{10} E + 1.20;$$

that is, the (pupil diameter, $\log_{10}$ glare intensity) curve is approximately

parallel to the pupil diameter, $\log_{10}$ background brightness curve. For large glare angles, β becomes very nearly equal to c , or

$$\log_{10} \beta = 0.5 \log_{10} E + 3.27,$$

which means that, under these circumstances, the slope of the (pupil diameter, $\log_{10}$ glare intensity) curve should be only about half that of the (pupil diameter, $\log_{10}$ background brightness) curve.

The point has been investigated in more detail for two subjects by taking sets of pupil diameter measurements at constant glare angle and a series

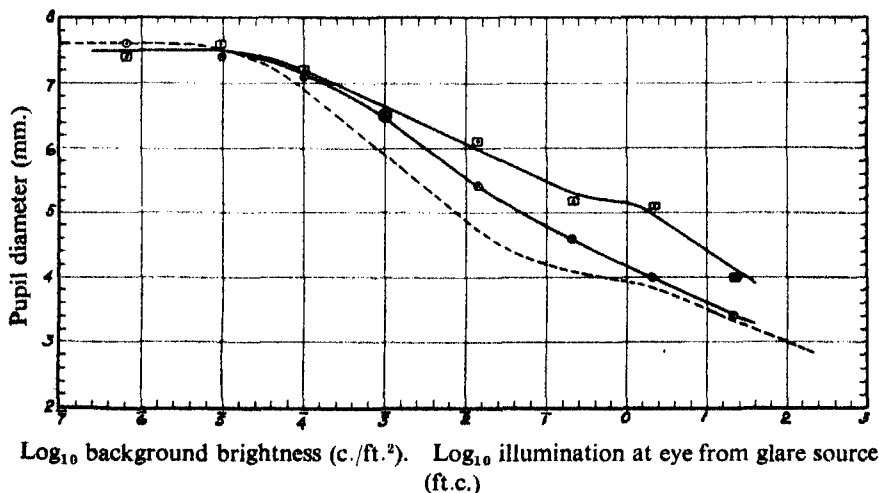


FIG. 3.—Variation of pupil diameter with background brightness and glare illumination. Subject, H. R. S. Background subtending solid angle of 0.37 steradians at eye, - - -; glare source at 5° from fixation point, ○; glare source at 30° from fixation point, □.

of glare intensities. The results are shown in figs. 2 and 3 and corroborate the conclusions which can be deduced from the data of § 1 (i). The following table shows values of maximum rate of variation of pupil diameter with $\log_{10}$ (glare intensity) for various conditions. In the last column the figures are reduced so that the values for a background or for a glare source directly fixated are equal to unity. The three groups of figures show approximately the same relative variation of slope with glare angle in each case.

TABLE III

Subject	Condition of eye illumination	$\left(\frac{dD}{d \log_{10} E}\right)_{\max.}$	
		Actual	Reduced
B. H. C.	Background	1.63	1.00
"	Glare source at 5°	1.05	0.64
"	" 28°	0.91	0.56
H. R. S.	Background	1.03	1.00
"	Glare source at 5°	0.91	0.88
"	" 30°	0.56	0.54
Mean data	Glare source at 0°	0.82	1.00
"	" 5°	0.71	0.87
"	" 30°	0.52	0.63

1 (iv)—*Variation of Pupil Diameter with Background Area at Constant Background Brightness*—The results of this part of the investigation are most profitably discussed in terms of equivalent background brightnesses; that is, a series of backgrounds of variable size and constant brightness are transformed into a series of backgrounds of constant (large) size and variable brightness giving the same series of corresponding pupil diameters. In view of the fact that as a glare source recedes from the fovea its equivalent background brightness diminishes, one would expect that with increase in size of a background the ratio of equivalent background brightness to background area would diminish steadily, the parts more remote from the fovea having less pupillo-motor effect. One can, in fact, assume that the variation of equivalent background brightness with glare angle represents the variation of specific pupillo-motor effect over the retina and so obtain by integration the effect of a background of any size. The results actually obtained do not seem to support this theory, except under very limited conditions. Figs. 4 and 5 show the data obtained for two subjects. The circles are experimental points, the dotted lines are the curves integrated from the (equivalent background, glare angle) curves for the respective subjects, and the lines at 45° to the axes are the integrated curves assuming uniform pupillo-motor effect over the whole retina. Considering the sets of curves in order, set I, for a background brightness of 550 c./ft.², indicates that for small sizes the integration from the glare angle effect is followed, while for larger sizes there is a complete breakaway to the case in which angle effect is absent. In set II, for a background brightness of 20 c./ft.², the experimental points agree throughout, within experimental error, with

the assumption of uniform pupillo-motor effect over the retina. The integrated curve allowing for variation of pupillo-motor effect shows no agreement with the experimental points. Set III, for a background

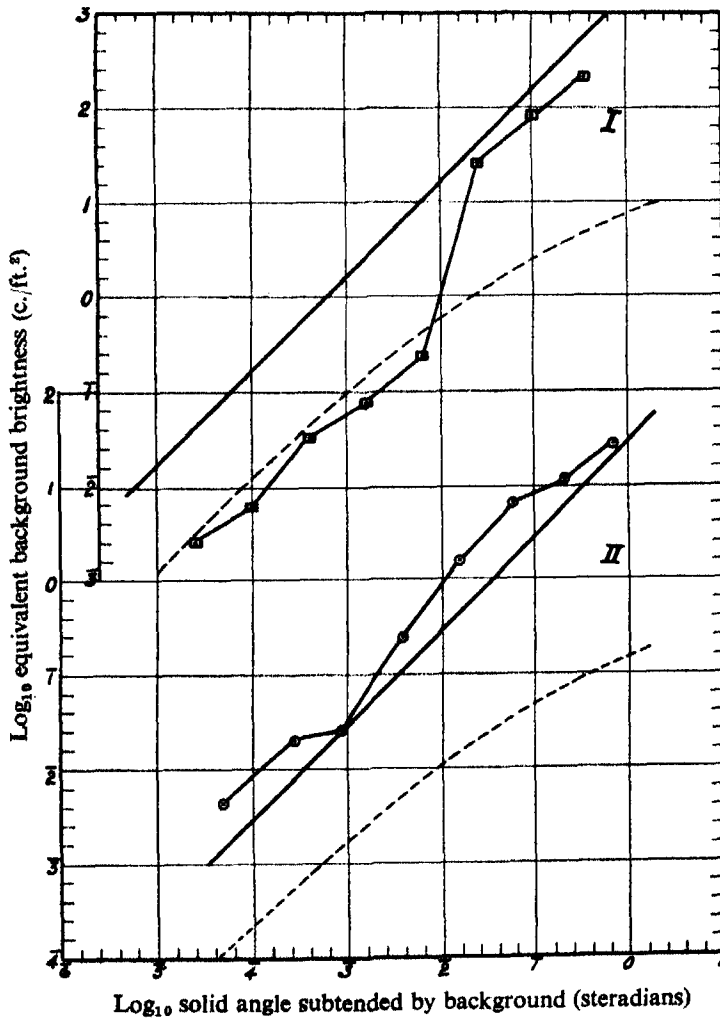


FIG. 4—Variation of the equivalent background brightness relating to pupil size with the solid angle subtended by the background at the eye. Subject, B. H. C. Curve I, background brightness = 550 c./ft.²; curve II, background brightness = 20 c./ft.².

brightness of 74 c./ft.², shows good agreement between experimental points and both theoretical curves for small background sizes, but at larger sizes the integrated curve allowing for variation of pupillo-motor

effect deviates considerably. Set IV, for a background brightness of 21 c./ft.², is difficult to interpret definitely, but there is no agreement between the experimental points and the integrated curve which allows for variation of pupillo-motor effect. The disagreement with the other theoretical curve may be only experimental error.

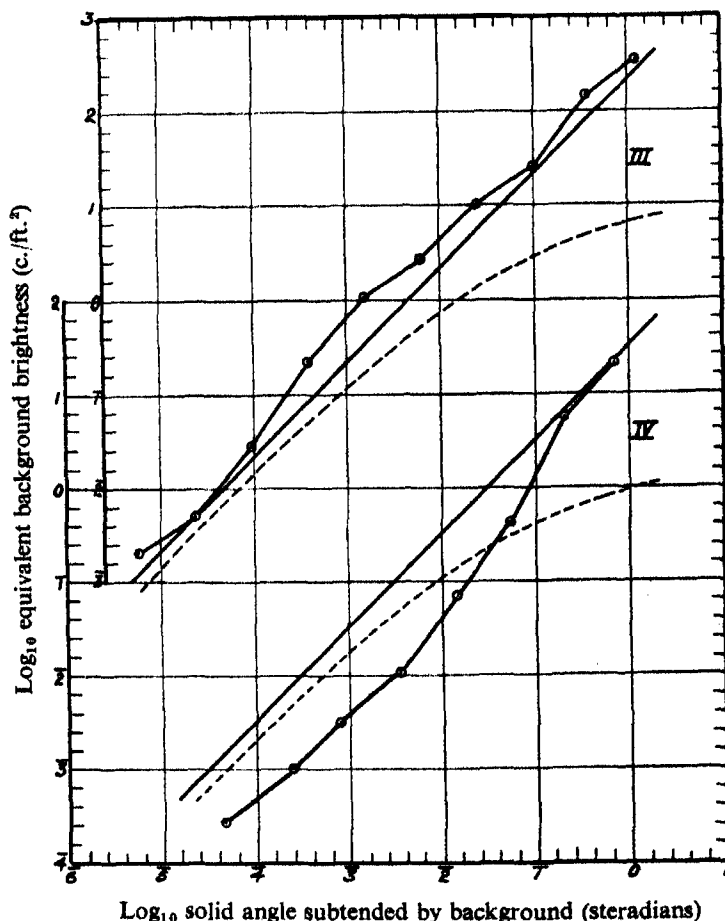


FIG. 5.—Variation of the equivalent background brightness relating to pupil size with the solid angle subtended by the background at the eye. Subject, H. R. S. Curve III, background brightness = 74 c./ft.²; curve IV, background brightness = 21 c./ft.².

The following general conclusions may be drawn from these results. For moderate background brightnesses the equivalent background brightness is proportional to the area. For high background brightnesses deviations occur when the area is small. It would seem that the pupillo-

motor receptors in the retina become less efficient with increase in intensity of the incident light, the effect being more marked the greater the distance from the fovea. This would explain the shape of the curves I, fig. 4, and the deviation between curves integrated from the glare angle effect and the actual results; the glare source used was small and of high brightness (a lamp filament) and therefore showed up in an extreme degree the falling off in efficiency of the pupillo-motor receptors with increase in intensity of incident light.

1 (v)—*The Addition of the Effects on Pupil Size of Background and Glare Source*—This question has not been investigated exhaustively as it is, in its most general form, a matter of some complexity. Such evidence as has been obtained, however, is not contradictory to the assumption that the equivalent backgrounds of the various components summate to give the equivalent background of all the components measured together. The condition tested was the addition of a uniform background subtending an angle of 43° at the eye and a glare source 10° above the fixation point. The equivalent backgrounds measured separately were 0.0030 c./ft.^2 and 0.0024 c./ft.^2 respectively. For glare source and background measured together the result was 0.0041 c./ft.^2 . The agreement between 0.0041 c./ft.^2 and 0.0054 c./ft.^2 is not good; on the other hand, the result demonstrates that no significant errors would occur in calculating pupil sizes from the assumption that a background and a few glare sources could be represented by the sum of the individual equivalent backgrounds.

1 (vi)—*Experiments with Ring-shaped Glare Sources and Backgrounds*—These experiments, somewhat deviating from the general track of the investigation, were carried out in an endeavour to throw more light on the variation of pupil size with background area and glare angle. In the first, a "glare source" was used in the form of a ring of variable diameter, fixation being at the centre of the ring. At each ring diameter the width of the ring was adjusted so that the illumination at the eye was constant, the brightness of the rings being also approximately constant throughout. These field patterns were obtained by projecting the images of diaphragms on to a screen in front of the subject. It was found that suitable diaphragms could be made with great facility from heavily fogged and developed photographic plates; the plates were mounted on a turntable and the requisite clear apertures cut out with a small, chisel-shaped, cutting point. The results obtained, for one subject, are shown in the following table.

TABLE IV—VARIATION OF PUPIL DIAMETER WITH SIZE OF RING-SHAPED GLARE SOURCE

Subject, H. R. S. Brightness of ring patterns = 10.5 c./ft.^2 .
 Illumination at eye = 0.123 ft. c.

Radius of ring (angle subtended at eye in degrees)			Pupil diameter mm.	Log ₁₀ equivalent background brightness c./ft. ²
Inner	Outer	Mean		
0	3.5	1.8	4.3	1.50
5.0	6.1	5.6	4.6	1.07
7.8	8.6	8.2	4.6	1.07
10.6	11.3	11.0	4.7	2.94
13.4	13.9	13.7	4.8	2.80
15.8	16.3	16.1	4.8	2.80

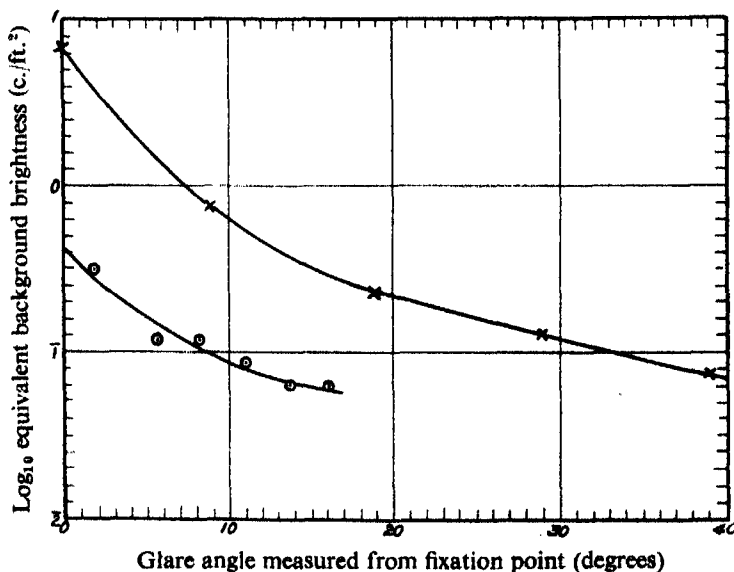


FIG. 6—Variation of the equivalent background brightness relating to pupil size with angle from the fixation point for an ordinary and for a ring-shaped glare source. Subject, H. R. S. Ordinary glare source, illumination at eye = 3.9 ft. c. , $\times$; ring-shaped glare source, illumination at eye = 0.123 ft. c. , $\circ$.

In fig. 6, these results are compared with the same subject's results for an ordinary glare source (a small projector lamp). It will be seen that essentially the same type of variation is present in the two cases, the rate of variation being smaller for the ring glare source. This, however, one would expect from the results of § 1 (i), since the eye illumination for the ring glare source is less than that for the ordinary glare source.

Some tentative conclusions may be drawn from the results of this experiment. The intrinsic brightness of the ring-shaped glare sources was low, so that the effect postulated in § 1 (iv), the falling-off in extrafoveal pupillo-motor efficiency at high stimulus intensity, would not be expected to occur. However, it does occur, and consequently it would seem that the pupillo-motor efficiency is a function of pattern quite as much as it is a function of intensity. The underlying mechanism may be localized in the retina or it may be of a psychological nature, that is, the direct pupillary light reflex may be modified by additional reflex actions

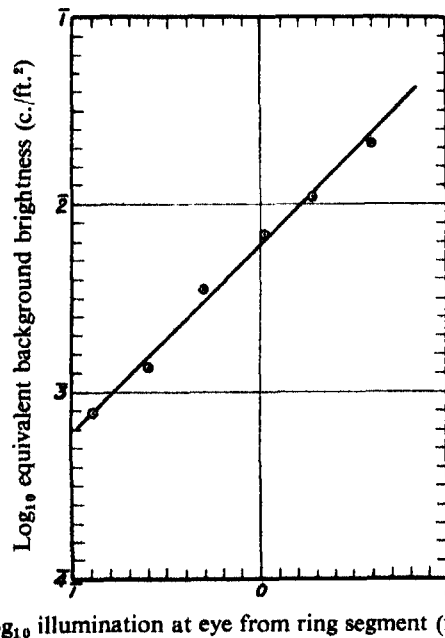


FIG. 7.—Variation of equivalent background brightness with illumination from background in form of segment of ring of variable length. Illumination is proportional to length of segment.

from the visual cortex depending upon the pattern presented to the subject's eyes. Of course, the latter explanation is very elastic and the present results are hardly capable of giving any definite pointer one way or the other; but it may be noted that all subjects find a glare source more unpleasant when viewed foveally than when viewed peripherally; thus, the necessary psychological stimulus is present and it appears to be well established that the necessary nerve connexions between visual cortex and pupillary musculature are also present.

A second experiment was carried out on the integration effect with a

background of variable size. In this the background was a segment of a ring, external radius 27.2° , internal radius 22.3° , fixation being at the centre of curvature. The variation in size took the form of variation in the length of the segment, the limits being from the complete ring down to about $\frac{1}{2}$ of the complete ring. The results are shown in fig. 7 for one subject, H. R. S. The experimental points agree well with the straight line drawn at 45° to the axes, indicating proportionality between equivalent background brightness and eye illumination from the ring segment. The agreement stops at proportionality, however, for if the equivalent background brightness gave the same eye illumination as the corresponding ring segment its value would be 240 times as great as actually found. Here again is apparent the falling off in pupillo-motor efficiency for a more or less small or isolated light pattern falling on the peripheral parts of the retina.

2—VARIATION OF PUPIL SIZE WITH TIME AFTER CHANGING FROM A HIGH TO A LOW LIGHT STIMULUS

The only data under this heading which appear to have been published are those of Reeves (1920). He measured the pupil size at intervals after reducing to zero an initial field brightness of 30 c./ft.². The measurements are probably quite reliable, but as they are unique they have been repeated and extended. The size of field used by Reeves is not stated, but it probably subtended a solid angle of about 1.5 steradians at the eye of the subject; this value is taken from an earlier paper by Blanchard (1918), whose work was apparently continued by Reeves using the same or similar apparatus. This would give a value of eye illumination of 45 ft. c. In the present work the initial conditioning field consisted of an opal lamp subtending an angle of 10° at the eye and of brightness 2700 c./ft.², giving the value of eye illumination of 65 ft. c. This is of the same order as Reeves's value, and the two sets of results are therefore comparable.

The opal lamp was placed within a box with a roller blind shutter so that it could be instantaneously cut off from view. Measurements were made on fourteen subjects selected at random. The mean curve for twelve of these follows the same general course as Reeves's mean curve (fig. 8, Table V). The degree of variation from the mean curve for the two investigations has been expressed as $\left(\frac{\Delta_m}{D}\right)_m$, where Δ_m is the mean variation from the mean for a given time, D is the corresponding pupil diameter, and the second suffix m indicates a mean of values over the range of the curve. The resulting figures are: for Reeves's data,

$\left(\frac{\Delta_m}{D}\right)_m = 0.77$; for the present data, $\left(\frac{\Delta_m}{D}\right)_m = 0.81$; values which are in tolerably good agreement with each other.

The time variation of pupil diameter for different initial pupil diameters is shown in fig. 9 for one subject. Apart from the irregularities, after about 100 seconds the curves converge regularly to the maximum pupil diameter from the starting diameters corresponding to the initial background brightnesses. The question of a possible dependence of the form of the (pupil diameter, time) curve upon the pattern of the initial stimulus has not been investigated, but it is probable that any effect would be small and not of any practical importance.

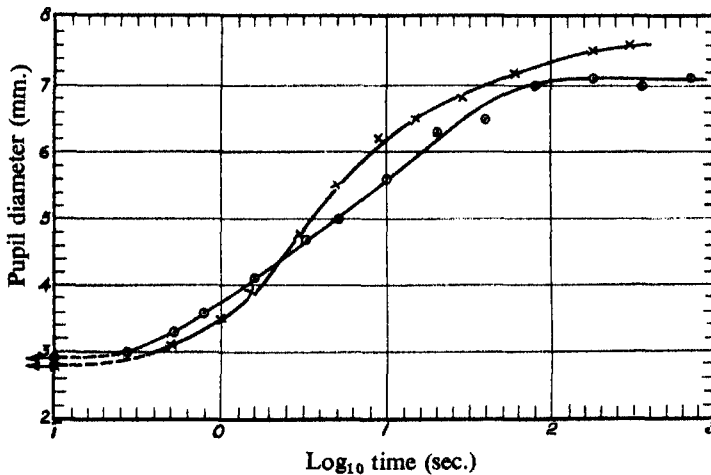


FIG. 8—Variation of pupil diameter with time after changing from high to zero background brightness. Present results, ○; Reeves, ×.

Two of the fourteen subjects tested, B. H. C. and F. M. H., showed very anomalous and curious behaviour of the pupil at about 5 minutes after cutting off the background field. The pupil contracted to some two-thirds of its maximum size for a minute or so and then expanded to the maximum size again. In order to investigate this effect more thoroughly, photographs were taken at half-minute intervals up to fifteen minutes, then at minute intervals up to half an hour. The use of infra-red photography completely avoided any interference with the pupillary reactions. The results of this method of investigation are shown in fig. 10. Subjects B. H. C. and F. M. H. consistently show a contraction of the pupil at about 5 minutes from the start, sometimes with, sometimes without further contractions at longer times. The extent of the first contraction is somewhat variable, so is the precise time at which it occurs. A small

TABLE V—TIME VARIATION OF PUPIL DIAMETER AFTER CHANGING FROM HIGH TO ZERO BACKGROUND STIMULUS.
PUPIL DIAMETER (MM.)

Time (sec.)	Subject														Mean
	W.B.	B.J.	W.O.	H.F.M.	H.B.	W.S.S.	G.E.V.L.	C.D.	F.J.C.B.	F.W.C.	G.M.N.	H.R.S.	G.W.G.S.		
0	2.4	2.8	2.8	2.8	2.7	3.0	2.6	2.6	3.1	2.7	3.1	3.3	3.6	2.9	
0.27	2.6	2.9	3.1	3.1	2.7	2.7	2.6	2.9	3.2	2.7	3.3	3.2	3.5	3.0	
0.53	2.8	3.2	3.4	3.4	3.2	3.1	3.2	3.3	3.4	3.0	3.5	3.6	3.8	3.3	
0.80	3.1	3.6	3.6	3.6	3.3	3.3	3.4	3.8	3.8	3.4	3.9	—	4.1	3.6	
1.6	3.5	4.3	3.9	3.9	3.6	4.0	3.9	4.1	4.3	3.9	4.4	4.5	5.1	4.1	
3.2	4.2	4.7	4.3	4.3	4.1	4.8	4.5	4.4	5.0	4.8	5.4	—	5.7	4.7	
5	4.5	5.5	4.0	4.0	4.5	5.3	4.9	4.6	5.0	5.0	5.4	5.9	5.2	5.0	
10	4.0	6.1	4.3	4.3	5.6	5.5	5.9	5.1	6.2	6.4	6.5	5.7	5.5	5.6	
20	5.0	6.8	6.6	6.6	5.7	6.6	6.0	5.9	7.1	5.7	7.1	6.4	6.2	6.3	
40	4.8	6.9	6.9	6.9	6.5	6.6	6.3	5.9	6.9	—	7.2	7.1	6.8	6.5	
80	5.0	7.4	6.8	6.8	6.5	6.6	7.1	6.6	8.3	—	7.6	7.6	7.7	7.0	
180	5.1	7.5	7.3	7.3	6.6	7.2	7.1	6.6	8.3	6.4	7.7	7.6	7.5	7.1	
360	4.2	7.0	7.6	7.6	7.1	7.2	7.3	7.0	7.6	6.4	7.9	7.6	7.6	7.0	
720	5.2	7.1	7.6	7.6	7.2	6.9	7.0	7.1	8.4	6.2	7.7	7.6	7.6	7.1	

effect seems to occur with a third subject, F. J. C. B., at or near 5 minutes from the start. For the other subjects tested, however, there is little or no indication of any contraction effect. The last curve, for subject F. W. C., is typical of these subjects. No explanation of this effect has been found, though it was noticed on some occasions that at the time of the contraction there was a vague feeling of difficulty in seeing dimly lighted objects in the room, the feeling of a shadow passing over them. The effect would appear to be present in only a minority of subjects, but the total number tested is not large enough to give any definite idea of the size of the minority.

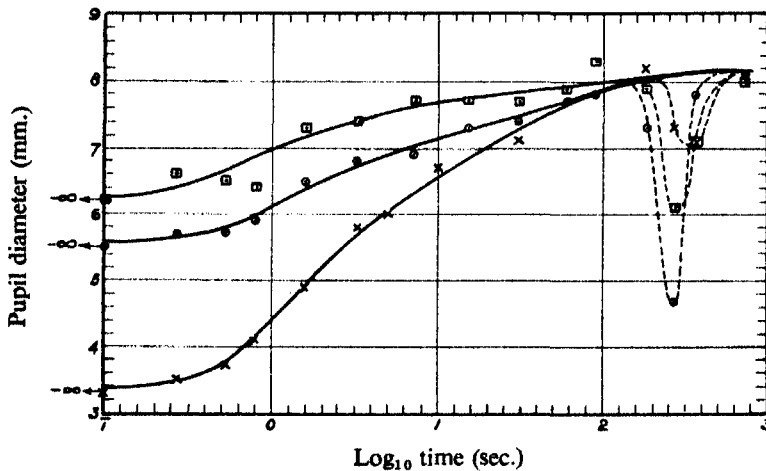


FIG. 9—Variation of pupil diameter with time after changing from high to zero background brightness for various initial brightness values. Subject, B. H. C.

SUMMARY

1—Measurements of pupil diameter have been made on a number of subjects under the following static conditions of illumination: (a) a single glare source at various positions in the field of view and giving various intensities of illumination at the eye; (b) a large uniform background at various brightnesses; (c) a uniform background of constant brightness and variable size; (d) a glare source and a background viewed together; (e) a "glare source" in the form of a narrow ring centred at the fixation point.

2—Measurements of pupil diameter for a number of subjects are given which were taken at intervals after changing a steady uniform field brightness to zero. Out of fourteen subjects tested, three showed a

curious anomaly in the form of a rather sharp contraction of the pupil after they had been about five minutes in the dark.

In conclusion, the author wishes to express his great indebtedness to all those who have acted as subjects for measurement, to Mr. H. R. Sayer

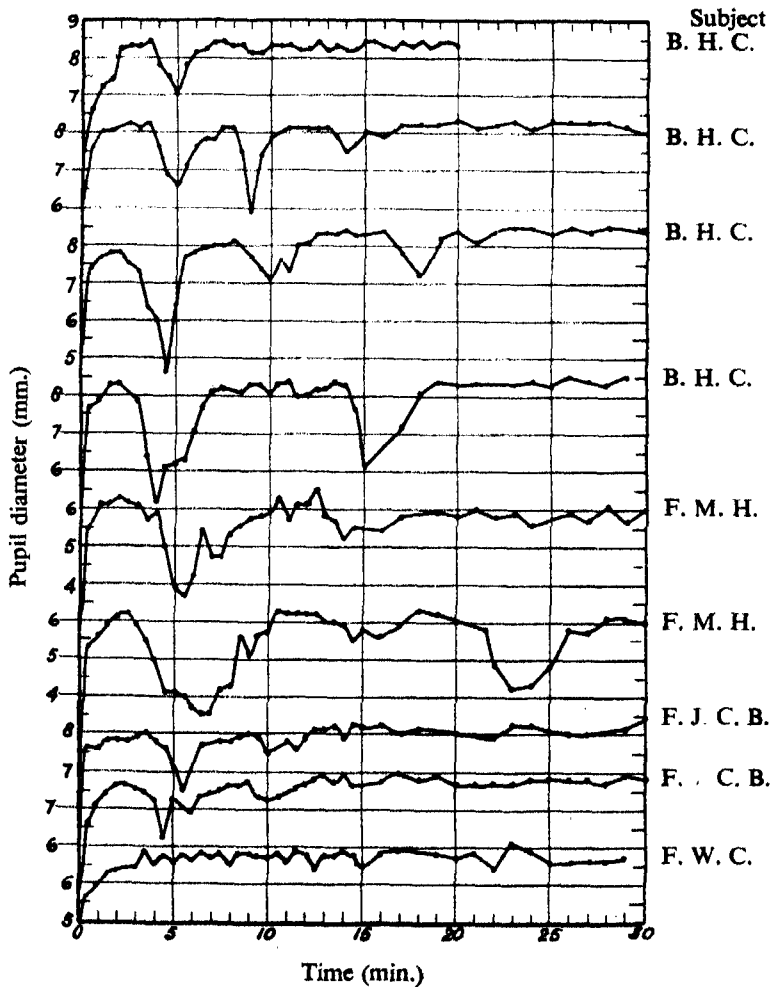


FIG. 10—Variation of pupil diameter with time after changing from high to zero background brightness, illustrating the contraction effect occurring spontaneously at various intervals after the start.

who has assisted throughout in the experimental work, and to Dr. W. S. Stiles for his suggestions and criticism during the course of the work, which has been carried out under the auspices of the Illumination Research Committee of the Department of Scientific and Industrial Research.

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Address of the President,
Sir William Bragg, O.M., at the
Anniversary Meeting, 30 November, 1936

The year which has passed since our last Anniversary Meeting is sadly distinguished by the heavy losses which death has brought upon our Society.

First and most conspicuous is the death of our revered Patron His Majesty, KING GEORGE V. With all his peoples we mourn the close of a life devoted to the cause of peace and progress. As members of our Society we feel deeply the loss of a Patron who was a true friend to the purposes for which our Society exists.

By the death of RICHARD TETLEY GLAZEBROOK, at the age of eighty-two years, we lose one of the most active and efficient scientific workers and organizers of his generation. Glazebrook began his scientific career under Clerk Maxwell and the late Lord Rayleigh at the Cavendish Laboratory. His first investigations were on double refraction. In the early 'eighties he became interested in the absolute determination of the electrical units, which continued to occupy him up to the very end of his life.

He was for a time Senior Bursar of Trinity and then Principal of Liverpool University, but on the establishment of the National Physical Laboratory in 1899, Glazebrook was appointed the first director, and the present prosperity and national importance of that institution are more due to him than to any other single man. It remains as an abiding monument of his life's work. His retirement in 1919 by no means marked the close of his activities. As chairman of the Aeronautical Research Committee, and of the Executive Committee of the Laboratory, he was still able to do much to promote the work to which he had devoted his best years. He was elected to the Society as early as 1882, and received the Hughes Medal in 1909 and the Royal Medal in 1931. He was created K.C.B. in 1920, and received numerous other honours. The debt which the country owes to him in the development of aeronautics is very great.

PERCY CARLYLE GILCHRIST was associated with his cousin, the late Sidney Gilchrist Thomas, in experiments which ultimately led to the establishment of the Basic Bessemer Process. Bessemer had discovered

in 1855 that a stream of air when blown through molten pig iron contained in a converter removed its carbon and silicon by oxidation, the heat evolved being sufficient to retain the metal in a molten condition. The metal thus produced was brittle owing to its oxidized condition. Within a year Mushet made the important discovery that if manganese was added to the molten metal in the form of ferro-manganese it removed the absorbed oxygen and enabled sound malleable ingots to be cast. These discoveries led to the establishment of the "acid" Bessemer process in which the lining of the converter is a siliceous refractory material. But phosphoric irons were not amenable to this treatment since with an acid lining the phosphorus remains in the finished steel and renders it brittle.

In the early 'seventies Thomas conceived the idea of lining the converter with a basic material and making additions of lime with a view to eliminating the phosphorus in the converter. After preliminary experiments he enlisted the help of his cousin, P. C. Gilchrist, who was then a chemist at Cwm Avon in South Wales. Experiments were continued at these works and Thomas and Gilchrist took out two patents. They were greatly assisted in their work by Mr. Martin of the Blaenavon Steel Works, who came to their assistance, and on 6 March, 1878, another patent was taken out. After this, progress was rapid and at the autumn meeting of the Iron and Steel Institute, held that year in Paris, they presented a paper entitled "On the Elimination of Phosphorus in the Bessemer Converter". The paper was not read at this meeting and was adjourned until the following one in 1879 when it was read by Thomas and published in the *Journal of the Institute*. In this way the Basic Bessemer Process was established and has proved to be one of the main processes for producing cheap steel on a large scale.

STEWART RANKEN DOUGLAS was Deputy Director of the National Institute for Medical Research and Director of the Department of Experimental Pathology and Bacteriology. He will always be remembered for his work, in association with Sir Almroth Wright, on opsonins and vaccines. The discovery of opsonins was communicated to the Society in 1903, a second communication followed early in 1904, and further work on these subjects during the following decade laid the foundations of vaccine therapy as it is practised to-day. Douglas devised several valuable nutrient culture media for pathogenic bacteria, and one of these proved invaluable during the War, for it enabled large quantities of vaccines to be made for the Army at a time when the constituents of culture media as then compounded were becoming difficult to procure. After the War, Douglas organized the new pathological laboratories at

the National Institute for Medical Research and devoted himself to the initiation of new lines of research and the encouragement of his juniors. He was largely responsible for planning and directing the scheme of study of virus diseases at the Institute which proved so fruitful. Although the publications under his name in this branch of knowledge are few, he inspired and guided much of the work published by his juniors. He was elected to the Fellowship of the Society in 1922 and to the Fellowship of the Royal College of Physicians in 1933.

JAMES HARTLEY ASHWORTH was a distinguished zoologist and acquired a wide reputation as a teacher, more particularly in that branch of the subject which is usually referred to as medical zoology.

In the early part of his career he made an important contribution to our knowledge of the Alcyonaria and afterwards turned his attention to the anatomy and systematics of the lug-worms (Arenicolidae). His elaborate investigation of the histology of the nervous system of these worms and the distribution of the giant-cells they possess, yielded results of great interest and importance.

Soon after his appointment to a lectureship in the University of Edinburgh, he was invited to provide a course on medical zoology for post-graduate students. As it was the first course of this kind to be given in this country, it was difficult at first to obtain a sufficient supply of specimens to illustrate his lectures; but with characteristic energy and skill he soon remedied the deficiency. In a few years the course acquired a wide reputation and attracted a large number of young medical men.

In connexion with this class, original research was encouraged and stimulated, his own great monograph on the organism *Rhinosporidium* which causes a polypus in the nose, being a contribution to our knowledge of outstanding importance.

Professor Ashworth will also be long remembered for the energy he displayed in obtaining the necessary funds for the magnificent new Zoological Laboratory in Edinburgh which now bears his name, and also for the wide knowledge and skill that he showed in expressing his wishes to the architects of that great building.

Professor IVAN PETROVITCH PAVLOV, best known of Russian men of science and greatest of Russian physiologists, died on 27 February last at the age of 86. He had been a Foreign Member since 1907, was Copley Medallist in 1915, and Croonian Lecturer in 1928. He was Nobel Laureate in 1904.

Pavlov was the son of a village priest and the grandson of a priest, and throughout his life, in spite of fame and position, he kept the simplicity of his origin, and in his teaching some of the attributes of the

priest. He preserved his vigour to the end, and in August, 1935, presided over an International Congress of Physiologists which his prestige, and the affection of physiologists for him, had brought to Leningrad. His position in Russia was unique among scientific men and unique in public estimation. The attention paid to science in the Soviet Union is due, to a significant extent, to Pavlov's character and achievements.

Pavlov's earliest researches were on the physiology of the circulation, and in that work he realized, as Starling said of him, the necessity of avoiding, if possible, disturbing factors such as anaesthetics, pain, and discomfort in experimental work on the normal functions of the body. From 1888 to 1900 came his great work on digestion, particularly on the nervous control of digestion. From 1902 onwards Pavlov and his pupils dedicated themselves to the problems of the higher nervous processes in the brain. The whole subject of "conditioned reflexes" was developed by Pavlov during that period. His demonstration that intelligent behaviour in animals is built up largely of conditioned reflexes, just as skilled movement is the integration of simpler reflexes, although its consequences may often have been exaggerated by others, will remain one of the fundamental contributions to the physiology of the brain.

Pavlov remained an inspired and inspiring teacher to the end. A few weeks before he died he wrote a "Bequest to the Academic Youth of his Country", which gives vividly the philosophy of his experimental work: "Never attempt to screen an insufficiency of knowledge even by the most audacious surmise and hypothesis. Howsoever this soap-bubble will rejoice your eyes by its play, it inevitably will burst and you will have nothing except shame". "Perfect as is the wing of a bird it never could raise the bird up without resting on air. Facts are the air of a scientist. Without them you never can fly. Without them your theories are vain efforts". "Do not allow haughtiness to take you in possession. Due to that you will be obstinate where it is necessary to agree, you will refuse useful advice and friendly help, you will lose the standard of objectiveness". "Remember that science demands from a man all his life. If you had two lives that would be not enough for you. Be passionate in your work and your searchings".

With Pavlov died one of the greatest of our Foreign Members, one of the greatest teachers and investigators in physiology, and a very good and simple man.

Professor CONWY LLOYD MORGAN, who died at the age of 84, entered the School of Mines with a view to becoming an engineer: here he came into contact with T. H. Huxley, under whose stimulating influence he acquired a deep interest in biology. After occupying a post in South

Africa, he was appointed lecturer on geology and zoology at University College, Bristol, succeeding Sir William Ramsay as Principal of the College in 1887. He had long had a deep interest in psychology and philosophy, and his sound scientific training well fitted him to take a broad synoptic view of the problems of life and mind. His researches on comparative psychology, especially on birds and dogs, were directed particularly to the segregation of innate and acquired factors in early behaviour, and are of permanent value in the development of a truly scientific psychology, founded upon a sound biological basis. In his later years he built up a philosophy of Emergent Evolution.

GEORGE THURLAND PRIOR, distinguished as a mineral chemist and a well-known authority on meteorites, was elected a Fellow of the Society in 1912. He was born at Oxford in 1862, and, after a school and university education there, he entered the Mineral Department of the British Museum in January, 1887. There he did his life's work. In 1909 he succeeded Sir Lazarus Fletcher as Keeper of Minerals, and on reaching the Civil Service age-limit he retired in December, 1927, after over forty years of service.

In addition to his work as curator of large and growing collections and to the labour involved in determinations and reports on specimens submitted to the Museum, he made many investigations on minerals, rocks, and meteorites. Many new minerals were based on his careful analytical work, often done on very small amounts of material. He excelled in the difficult analyses of minerals containing niobium, tantalum, titanium, zirconium, and the rare-earths; and problems relating to these were often referred to him by workers in other countries. One of these minerals, a titano-niobate of yttrium and cerium earths from Swaziland, which he had analyzed, was named *priorite* after him by W. C. Brögger, a Foreign Member of the Society. His petrographical work included descriptions of the rocks collected by the Antarctic expeditions of Ross (1839-43), Borchgrevink (1898-1900), and Scott (1901-04), and of alkali-rocks from Abyssinia and East Africa. In his work on meteorites he described many new falls and devised simpler methods for the partial analyses of others previously known. From his results he was able to show that "the richer in nickel is the nickel-iron, the richer in ferrous iron are the magnesium silicates". This may well be called Prior's Law. On it he based a new genetic classification of meteorites. His "Catalogue of Meteorites" is the standard work of reference. Dr. Prior was for eighteen years the General Secretary of the Mineralogical Society, and its President in 1927-30.

To the physiologist the name of JOHN SCOTT HALDANE will always be

associated with that masterly investigation of the respiration which was his outstanding contribution to pure physiology, for this has played a remarkable part in moulding the change of outlook in physiological thought which has been apparent since the beginning of the present century. Deeply interested in a philosophy of life which guided, and was in turn guided by, his own experimental researches, he has made clear by his work the exquisite and quantitative coordination of the different functions of the body on which the integrity of the living organism depends, and the delicate adaptive changes which are characteristic of life. By the introduction of new experimental methods and the design of special apparatus, he has shown how it is possible to use delicate methods of chemical and physical analysis to gain an insight into the physiology of the intact and normal human being. Far-seeing and original in his ideas and courageous in maintaining his views, his work ranged over a far wider field than academic physiology, for he drew no distinction between pure and applied science but found inspiration for his work in both alike. Much of his time and thought was devoted to investigations whose object was the reduction of the risks which the miner has to run in the course of his daily occupation, and the elimination of the diseases and discomforts associated with mining or with other occupations when men have to face foul air or extremes of temperature. By the application of strict scientific methods, he has rendered negligible the dangers which were formerly associated with deep diving and work in compressed air. Great as were his contributions to physiology his investigations in the field of general industrial hygiene were no less important and fruitful.

PERCY FRY KENDALL was born in London in 1856. He began his geological studies at evening classes, and in the early 'eighties took the full course at the Royal College of Science, South Kensington. He then went to Manchester as a Berkeley Fellow of Victoria University. His life's main work was the building up of the School of Geology at Leeds. Starting under difficult conditions as a part-time lecturer at the Yorkshire College in 1891, his teaching attracted students to his classes and brought prestige to the College, so that when Leeds University received its charter in 1906 he became the first occupant of the Chair of Geology.

Among geologists, Kendall will be remembered as the great protagonist of glaciation of Britain by land ice. He was the central figure in the organization of local workers who, through a British Association committee, made systematic records of the dispersion of glacial boulders. An outstanding contribution to natural knowledge is the demonstration of ice margin-retreat stages round the British hills, which was started

with his publication in 1902 on "The Glacial Lakes of Cleveland" in the *Quarterly Journal of the Geological Society*.

From about 1900, Kendall's attention was directed to the supply of underground water for Yorkshire towns and villages, and so to the problems of the coalfields. For the Royal Commission on Coalfields in 1905 he produced a masterly report, documenting the local application of the doctrine of posthumous folding, and estimating the unproved area of the Yorkshire, Nottinghamshire, and Derbyshire coalfield at 3885 square miles.

Professor Kendall was awarded the Lyall medal of the Geological Society in 1909. He retired from the Chair at Leeds in 1922 and was granted the title of Emeritus Professor. He was elected into this Society in 1924, and received the honorary degree of D.Sc. at the coming of age of Leeds University in 1926. He died at Frinton-on-Sea in March, 1936.

By the death of Sir ARCHIBALD GARROD, the Society loses a Fellow who, while primarily an investigator, became famous as a practising physician and pathologist. He was trained in medicine at St. Bartholomew's Hospital. Early in his career he was attracted by the subject of urinary pigments, and published papers on haematoporphyrin, urochrome uroerythrin, and urobilin. His work on alkaptonuria was of primary importance: he saw in it, not a disease but an individual variation; these and other studies led to the publication of his book on "Inborn Errors of Metabolism" in 1909. His professional career absorbed much of his time, and left him little leisure for personal research, though his sympathies and interest were a direct stimulus to much valuable work while he was Regius Professor of Medicine at Oxford from 1920 to 1927. He was elected a Fellow in 1910 and was a Vice-President from 1926 to 1928.

Sir JOSEPH ERNEST PETAVEL was born in London in 1873, spent his early years at Lausanne where he studied engineering and returned to England in 1893 to continue the study of his subject for three years at University College, London. For the next three years he worked in the Davy Faraday Laboratory of the Royal Institution on the properties of matter at low temperatures, on the emission of light and heat from carbon and platinum with the object of obtaining a standard of light; there also he devised the well-known "Petavel gauge" for measuring the rate of rise of pressure in explosive reactions.

Proceeding to Manchester University in 1901, he lectured on mechanics and meteorology and applied his conspicuous gift for design to the evolution of a new technique for work at high pressures and temperatures. This had an important influence on the study of ballistics, and of com-

bustion, and on the industrial development of chemical reactions under the above conditions. He became Professor of Engineering there in 1909.

Through his interest in the problems of the upper air, he came into touch with aeronautics, and in the early days of the War supplied a definite stimulus to the study of aerodynamics, resulting in improvement in the stability of aeroplanes. His fertility in design was also employed in other work for the Admiralty and the War Office.

In 1919 Petavel succeeded Sir Richard Glazebrook as Director of the National Physical Laboratory and gave his undivided attention to the extension of its activities and especially to the increase of its usefulness to industry.

Petavel will be remembered not only for his achievements in pure science and in administration but also for his personal qualities, his courtesy, hospitality, and love of his house and gardens, for the upkeep of which he expressed a wish that a contribution should be made from his bequest to this Society.

Professor KARL PEARSON, Galton Professor of Eugenics at University College, London, from 1911 till his resignation in 1933 at the advanced age of 76, died suddenly on 27 April. Elected to the Goldsmid Professorship of Applied Mathematics at University College in 1884, he began the statistical work with which his name is chiefly associated about the year 1891, his first statistical memoir being printed in the 'Philosophical Transactions' of 1894. From then onwards there followed an ever-increasing mass of work at first largely in our 'Transactions' and 'Proceedings', later mainly in the journal 'Biometrika' which he founded in 1901, work which may well be said to mark a new epoch in the history of statistical method. Pearson was not only himself an indefatigable worker, but an outstanding teacher with a great capacity for rousing enthusiasm in others, and his pupils are scattered all over the world. But he was not only a statistician; he wrote on other branches of applied mathematics and in earlier years edited and completed Todhunter's "History of the Theory of Elasticity." This, the well-known "Grammar of Science," the "Life of Francis Galton," and several of the essays in "The Chances of Death and other Studies in Evolution," fall within the field of science. An early little book on the Veronica legend and portraits of Christ, and the essays of "The Ethic of Freethought" show the scholar, historian, and philosopher. Elected a Fellow in 1896, Pearson was awarded the Darwin medal in 1898, but never took any active part in the general work of the Society.

ALFRED CARDEW DIXON, who died on 4 May last at the age of 70, was

Senior Wrangler in 1886, Fellow of Trinity, and afterwards Professor of Mathematics successively in Queen's College, Galway, and the Queen's University of Belfast. Like most Cambridge mathematicians of what is now the older generation, his interests extended over a very wide domain in the science, and at different times he published original papers on geometry, analysis, dynamics, and the theory of elasticity. Among his best-known achievements may be mentioned his theory of the singular solutions of systems of differential equations of any order; his papers on the integration of partial differential equations; his great memoir of 1901 on matrices of infinite order—a subject in which he was a pioneer, and a subject now of great importance in the last decade on account of its applications in quantum mechanics—and the researches of his later years on the theory of integral equations and the problems of the elastic plate. As a teacher and administrator he was most highly regarded in the University of Belfast, which conferred on him the honorary Doctorate of Science. After retirement from his chair in 1930, he settled near London and took an active part in the affairs of the London Mathematical Society, of which he was President in 1932–33.

Sir GEORGE HADCOCK, a Fellow of the Royal Society since 1918, was a man of great charm of manner, and was much liked by all with whom he came in contact.

His extensive experimental work in connexion with the development of artillery in all its phases was largely responsible for the present position of the science of gunnery and ballistics; and his “Ballistic Tables,” published in 1897, were for many years the official tables of the War Office to be used in conjunction with the “Text-book of Gunnery.”

Hadcock conducted experiments to determine the effect on the resistance of thick-walled cylinders to internal pressure, which were some of the first to be carried out in this country; and upon them our present knowledge of overstrain in metals is largely based.

He was the author of several books and many papers on Artillery and Ballistics, and wrote articles on these subjects for the “Encyclopaedia Britannica.” He also contributed a paper, which was published in the ‘Proceedings of the Royal Society,’ on the “Longitudinal Strength of Cylinders, etc.”

Professor WILLIAM ERNEST DALBY, Emeritus Professor of Engineering in the University of London, died on 25 June, 1936, at the age of 73. At the early age of 14 he commenced his practical training at the Stratford Works of the Great Eastern Railway. Then, having been awarded a Whitworth Scholarship, he went to the Crewe Works of the London and North-Western Railway. In 1891 he accepted an invitation from

Professor Ewing to act as his assistant and from that time onwards his career ran along academic lines.

After six years with Ewing, Dalby was appointed to a Professorship of Engineering at the Finsbury Technical College, where he remained from 1896 until 1904, when he was promoted to the Chair of Civil and Mechanical Engineering at the City and Guilds College at South Kensington. This Professorship of London University he retained until his retirement from academic work in 1931. Although Dalby in no wise neglected or belittled his duties as a teacher, his interests were mainly concentrated on research, particularly research of a practical variety. His researches were very varied and, with the exception of the electrical, dealt with almost every branch of engineering knowledge. When at Finsbury the research which monopolized his attention was concerned with "Balancing of Engines". On this subject he became recognized as the highest authority. It was his first love in research and it is the one with which his name will continue to be associated by future generations of engineers. Elected a Fellow in 1913, he served on the Council during the year 1924. Hard-working, conscientious, and conspicuously generous minded in his estimate of others, he leaves behind him the pleasing memory of a man who without motive invariably gave the best that was in him to the cause of engineering science and the betterment of engineering education.

RICHARD DIXON OLDHAM, who died on the 15 July, 1936, at the age of 78, spent 25 years of his active life of research as a member of the Geological Survey of India; and after his retirement from official work in 1904 he continued to carry on, with marked distinction, his studies of seismology and physical geography. His work in India was mainly devoted to the regional geology, and especially the stratigraphy of Baluchistan, the Himalayas, Northern Peninsular India, and the Andaman Islands. This wide range of field experience qualified him eminently for revising the official Manual of the Geology of India, which was published in 1893.

In a paper, published in 1906, analysing the seismographic records of fourteen world-shaking earthquakes, Oldham established the existence of two distinct sets of deep-seated waves, travelling at different speeds; and, from the way in which the waves of distortion were damped out in depth, he deduced the existence of a central core in the earth, four-tenths of the radius in thickness, which contrasted in physical properties with the external shells. In this way Oldham pointed to the pretty analogy between seismic waves as a source of information regarding intra-telluric conditions and those of light which, on analysis by the spectroscope, give information regarding the composition of the sun's atmosphere.

Oldham was elected a Fellow in 1911 and served on the Council of the Society in 1920-21. He was also elected President of the Geological Society of London in 1920, and in 1931 an Honorary Fellow of the Imperial College of Science in which he received his early training.

Sir HENRY WELLCOME, head of the great firm of Burroughs, Wellcome & Co., died in London on 25 July, at the age of 82. Famous throughout the world for his commercial achievements, he attained still greater renown for his lifelong interest in medical science, to the promotion of which, in one or other of its branches, he devoted most of his energies and practically all his wealth.

After an early training in pharmacy and chemistry in America, where he was born, he came to this country and founded with Burroughs, in 1880, the firm which later passed into his sole control.

In 1894 in the early days of serum therapy, he grasped the possibilities of protective and curative sera and founded the Physiological Research Laboratories with the twofold object of bringing serological and biological remedies within the reach of medical men and their patients and of carrying out researches into the fundamental problems of immunology and allied subjects. Two years later he founded the Chemical Research Laboratories, and then in 1913 the Bureau of Scientific Research to carry out researches in tropical medicine and coordinate the work of the various research laboratories and museums he had founded. The museums included the Museum of Medical Science depicting modern medicine in graphic form and the Historical Medical Museum illustrating, by a most extensive and priceless collection of instruments, objects, and books, the history of medicine from the earliest times to the present day.

He built the Wellcome Research Institution in the Euston Road, and established the Tropical Research Laboratories in Khartoum.

He made arrangements in his will for the continuance of his great business and of his various research laboratories and museums, all of which are now grouped under the name of the Wellcome Foundation. Over and above this, residuary profits are also to be devoted for the most part to the furtherance of scientific research.

Dr. BERNARD SMITH died on 19 August, having held for only ten months the Directorship of the Geological Survey of Great Britain and of the Museum of Practical Geology, London. He had served on the Council of the Royal Society for nearly a year and was chairman of a committee on Ordnance Survey Maps. For thirty years he had been a member of the Geological Survey. He had been since 1931 Assistant to the Director, and in charge of field work in England.

Smith was essentially a field geologist with a special bias to the study

of the relations of physiography to geology. This was well shown by his published text-book of *Physical Geography*, which is in its third edition. His principal contribution to British geological science was his interpretation of the Whitehaven coalfield. This difficult ground was entrusted to him, and a staff of geologists working under his direction, in 1920, and the maps and memoirs are now nearly all published. The work maintained a very high standard and solved many problems previously little understood. Almost equally important was his investigation of the iron-ore deposits of West Cumberland and Lancashire, the principal sources of British haematite.

Smith's early death cuts short a career of great promise. His ability was shown in all his investigations and his judgment was cautious, sound, and penetrating. With equal ease he handled a great variety of subjects and as a colleague and director he was universally esteemed. Gentle and unobtrusive in manner, he won the confidence of his colleagues and of the public, and, unspoiled by popularity and success, he exerted a stimulating influence on wide circles of British geologists.

HENRY LOUIS LE CHATELIER devoted almost the whole of a long and active life to physical chemistry in its applications to concrete problems, giving special attention in the later years to those of metallurgy. His early work on the dissociation of calcium carbonate led him to the enunciation of the "Le Chatelier principle", which indicates the influence of a change of external conditions on the state of a system in equilibrium. This principle has played a great part in the development of physical chemistry. His application of the theory of solutions to metallic alloys opened up a new field, which has proved very fertile. He was an excellent experimenter, and introduced several new instruments, the most important of which was the thermo-electric pyrometer, universally used in the study of alloys. By his foundation of the '*Revue de Métallurgie*' in 1904 he provided a medium for the publication of French researches in metallurgy, which has ever since retained its position as a leading technical journal. The esteem in which he was held by scientific men of many countries was shown by the celebration in Paris of his academic jubilee in 1922. A graduate of the *École des Mines*, and for many years one of its professors, he approached chemistry from the side of a mining engineer, and this fact determined his practical bias. Both by his teaching and through his important text-books he exerted a great influence on the development of chemistry and chemical industry in France. He was elected a Foreign Member of the Royal Society in 1913.

WILLIAM JOHNSON SOLLAS, who was elected a Fellow in 1889, died at the age of 87, retaining undiminished his remarkable energy and many-

sided interest in science. His researches not only illuminated his own special province of geology with mineralogy, but also included important contributions to zoology and anthropology, even, in his work on the *Age of the Earth*, venturing into the domain of physics. He was educated at the City of London School and the Royal School of Mines, and wrote, not many months ago, his memories of student days under the inspired teacher T. H. Huxley, and of the group of friends who, led by the late Dr. William Garnett, migrated to Cambridge. After many years of teaching his subject in Dublin, he became in 1897, and remained until his death, Professor of Geology at Oxford, where the arrangement and great development of the University collections and the deepening interest in geological study will be long remembered as the fruits of his occupancy of the Chair.

Sollas was formerly Fellow of St. John's College, Cambridge (elected 1882), and became Fellow of University College, Oxford, a few years after his election to the Professorship. His election last year to the honorary Fellowship of the Imperial College of Science gave him much pleasure. He was President of the Geological Society of London 1908-09, and was awarded a Royal Medal in 1914.

Professor GEORGE FORBES was a man of great versatility and was particularly interested in Physics, Astronomy, and Electrical Technology. He was born in 1848. After graduating at Cambridge he was appointed, 23 years of age, Professor of Natural Philosophy in Anderson's College, Glasgow, which post he held until 1880. One of his chief pieces of physical work was a determination of the velocity of light by a modified Fizeau method carried out in conjunction with Dr. T. Young and described in 'Philosophical Transactions' in 1882. The result obtained was a velocity of 301,382 kilometres per second. In 1874 he took charge of an expedition to Hawaii to observe a transit of Venus.

When incandescent electric lighting began commercially at about 1882, Forbes came to London to occupy a post in an electric manufacturing company and made improvements in arc lamps and electric meters. His chief contribution was the introduction of brushes for dynamo machines and motors made of hard graphite carbon; an improvement of general utility. When the great project of employing part of the water power of Niagara for generation of electric current was started, Forbes was appointed to superintend the work of the electric installation and plant erection at the site and acted as consulting engineer to the Cataract Company carrying out the work.

Forbes travelled extensively and was a correspondent of 'The Times' during the Russo-Turkish War. He invented a naval gunsight and a

military range-finder and was granted a Civil List Pension in 1931 for his services.

He was elected a Fellow in 1887 and was a Chevalier of the Legion of Honour of France and an Honorary Member of the Franklin Institute of America. He died at Worthing in October, 1936.

With the death of WILLIAM ARTHUR PARKS on 3 October, the Society loses a great Canadian and a great scientist. He was born at Hamilton and graduated at the University of Toronto, in which he was afterwards Professor of Palaeontology. He joined the geological staff of the University in 1893, and touched on nearly every aspect of geology during his career. His exploration of Northern Ontario broke new ground, and his report on the building and ornamental stones of Canada is well known. But his favourite subject was undoubtedly palaeontology, and to the world outside Canada he will be remembered chiefly for his classical work on dinosaurs. He became director of the Royal Ontario Museum of Palaeontology in 1913; in 1926 he became President of the Royal Society of Canada. He was elected to our Fellowship in 1934.

THOMAS MARTIN LOWRY, Professor of Physical Chemistry in the University of Cambridge, died on 2 November, 1936, at the age of 62. In his early days he was a pupil of, and an assistant to, Professor H. E. Armstrong and laid the foundation of his lifelong studies on optical rotatory power by discovering the mutarotation of nitrocamphor and the stereoisomerism of a number of halogen derivatives of camphor. He traced the mutarotation of nitro-*d*-camphor to the passage of equilibrium of two constitutionally different forms of the substance, and showed that the rate of change of the one form to the other could be influenced by the addition of traces of catalytic agents. By much careful work he established that the presence of an amphoteric agent is a factor in bringing about the isomeric change. Thus, whilst the mutarotation of tetramethyl-*d*-glucose can be arrested in the hydroxylic solvent, cresol, and the basic solvent, pyridine, it proceeds very rapidly in a mixture of these two solvents. On the basis of this and much other work, Lowry founded his now well-known theory of *prototropy*, according to which the migration of a hydrogen ion, in compounds such as nitrocamphor and the sugars depends on the addition and removal of a proton at opposite poles of the molecule. It is largely due to Lowry's work that the conception of dynamic isomerism advanced by van Laar became generally accepted.

Concurrently with his chemical work on mutarotation, Lowry took up the study of optical rotatory dispersion which had been much neglected since the death of Biot in 1862; he demonstrated the validity of Drude's equation for simple substances and showed that it also covered the

anomalous rotatory dispersion of *d*-tartaric acid and the tartrates. This work formed the subject of the Bakerian Lecture given by Lowry and Austin in 1921. His later very precise determinations of the rotatory power of quartz in the visible and violet provided valuable data by which the validity of the Drude equation was further extended.

Lowry later extended his studies of the rotatory power of transparent media to that of absorbent media, namely of the Cotton effect, and was able to develop equations which are adequate to express the dispersion throughout the absorption band. Whilst occupied with the study of optical rotatory power, Lowry carried on parallel lines of research; his earlier verifications of Drude's equations were combined with corresponding measurements of magneto-rotatory dispersion and, in his search for possible relationships between diverse optical phenomena, he carried out a series of investigations on the refractive dispersion of organic compounds.

The Council's Report for the year is now in the hands of the Fellows. I propose to refer to one or two of its statements.

In the first place we are all happy to read in it the gracious letter which we have received from His Majesty the King, intimating his consent to become our Patron, and his pleasure in continuing the long-established connexion between the Crown and the Society.

It is also a source of gratification to the Society that His Majesty has promised to continue the annual grant of two Royal Medals.

Three magnificent gifts have been made during the last few months for the furtherance of research in this country. In May it was announced that Lord Austin had placed a quarter of a million sterling at the disposal of the University of Cambridge to be devoted to the work of the Cavendish Laboratory. More recently still Lord Nuffield has presented to the University of Oxford two millions for post-graduate research in medical science. This afternoon the Council of the Royal Society has accepted on behalf of the Society a principal share in the responsibility for the administration of a sum of £200,000 bequeathed by the late Mr. H. B. Gordon Warren. The interest of this money is to be applied to the encouragement of research in metallurgy, engineering, physics, and chemistry. The administering committee is to consist of eight members appointed by the Society and two by the Governors of Williams Deacon's Bank.

These great gifts are naturally a source of deep satisfaction to the Fellows of a Society which was founded for the purpose of "Improving Natural Knowledge". The givers are men who are or have been

engaged in industry: which fact is itself a source of gratification. It is good to see that practical affairs are in accord with the realization of the vital importance of research.

Lord Nuffield's gift recognizes the value of research for the prevention and cure of disease. The obvious success which has already been attained in this way is sure ground for the expectation of further benefits commensurate with the magnitude of the new effort. Lord Austin's gift is for the promotion of investigations which are at the time devoted mainly to the abstruse problems of the atomic nucleus. The terms of Mr. Gordon Warren's bequest suggest a more immediate contact with industry.

Thus these benefactions differ widely in respect to their immediate purposes: but they all acknowledge the same principle, that the improvement of Natural Knowledge is essential to the general welfare. As for our Society, it is the basis of its Charter and the reason for its existence.

The capital value of the funds administered by the Society, if we include in them the Warren bequest, is now more than a million sterling. In this amount is also included the bequest of about £40,000 by the late Sir Joseph Petavel, to which reference is made in the Report of the Council. By far the greater part of the money has been received within the last twenty years. In 1828 Dr. Wollaston founded the Donation Fund, the first fund of which the income was to be devoted to research; the amount was about £3400. By the beginning of this century there were several such funds, the combined income of which was £1375. In 1912 the total income had risen to a little over £2000 a year. Then in 1919 began a period of large donations. First came the Foulerton Gift and the Foulerton Bequest. The Messel bequest was received in 1921. The Yarrow and the Mond Funds came in 1923, the Medical Research Fund in 1924 and 1925. To these the Warren bequest has to be added. In all the Society now directs the expenditure of about £31,000 a year on research. The direction makes a considerable demand upon the time and energies of Fellows, and it is a pleasant duty to acknowledge their willing and able service on numerous committees.

The use to be made of these moneys is to a considerable extent limited by the terms of the respective trusts. Nevertheless, there is ample opportunity for a general policy at the discretion of the Society. It is natural and right that special emphasis is laid upon general or fundamental research, so far as donors' wishes allow; and indeed the terms in which the donors have expressed themselves are favourable to research of that kind.

It is to be observed that many other bodies possess funds which are

administered for similar purposes. In a list published by the Royal Commission for the Exhibition of 1851, the Commission itself takes place as one of the oldest, and the Leverhulme Trust as one of the newest. The list includes such well-known names as the Carnegie Trust, the Halley Stewart Trust, the Beit Memorial Fellowship Trust, and others. City companies are also to be found there. The Improvement of Natural Knowledge follows also on the activities of many bodies that have specific applications in view. Each branch of the Defence Services maintains its own research laboratories; so do the Medical Research Council, the Department of Scientific and Industrial Research, the Agricultural Research Council, the Post Office, and so on.

Still more closely concerned with the direct applications of Natural Knowledge are the laboratories of the country's industries. Many of these are of great and established reputation. On the whole, the industrial laboratory is some way from being as frequent a factor in industry as it ought to be, but undoubted progress has been made in recent years.

This brief enumeration of some of the agencies making for the improvement of natural knowledge will serve as a reminder that the sum total of the work done in this direction is very large. It may fall far short of what is to be hoped for, but it forms an agency which begins to acquire a certain coherence, something which can be viewed as a whole and considered in respect to its character and its effects. It is beginning to find itself, like Kipling's ship.

An immediate and obvious effect is the increase in the volume of published results. The publications of scientific societies have doubled and trebled in size; and their treasurers are in many cases hard put to it to meet the consequent additional expense. Numerous industrial publications also contain records of special investigations. There is every reason for satisfaction with the increase in natural knowledge which has followed on the encouragement of research.

In certain respects, at least, the application of the knowledge acquired is also satisfactory, though judgment on that point will vary according to the position of the observer in a very large field. There are obvious improvements in the health and general well-being of the nation, in its industries, in the strength of its trade, and in its powers of defence; and these are matters of primary importance. Though they may be no more than means to an end, they and the appropriate application of knowledge are a first consideration.

To such applications every kind of research may contribute; for even those who would have it that Science must be followed without thought of its usefulness must admit that it has to be very pure Science indeed

which only meets with its application, as a straight line meets its parallel, at infinity. In general the encounter may be expected to come so soon that its effect has a present importance, and must be taken into account. The individual member of the Society may keep his thoughts and his experiments within an isolated region, and so contribute what is due from him as a Fellow. But the Society as a whole must take the wider view, and watch constantly the relations between scientific advance and the people who are affected by it. It accepts these responsibilities when it undertakes to administer the great sums that have been entrusted to it. In the early days of the Society the Fellows recognized duties in these respects, as the records of their Transactions show. Many of the Founders occupied important positions in the State and their science bore directly on the needs of the nation. Throughout the three centuries of its existence, the same ideals have encouraged the activities of the Society. At some times they have been less effective than at others, but their general purpose has never been blurred. The whole of the work of the Society is therefore an important part of a general effort to improve natural knowledge in the expectation of resultant benefit.

Another consequence of that effort deserves especial consideration. The increase of knowledge and its applications are, each in its own way, worth working for. At the same time there should follow, and does follow, an increase in the quality and quantity of men who can add to knowledge and use it; also, it may be hoped, an increase in the number of those who realize its effectiveness. This is an exceedingly important point. It might seem unnecessary to observe that the resources which a nation possesses are of no use unless there are the foresight and the skill which are needed to make use of them. Yet a nation as a whole might fail to act on a principle to which its individuals would give a ready assent. The principle has to be stated plainly, so that it may be widely understood. One of the greatest assets of a nation is the presence within it of men who are quick to apply the knowledge of the time to the needs of the time. There are many varieties of such men. There are the handicraftsmen, whose skilled fingers are guided by intelligence; such skill is steadily increasing in this country, though the contrary is often asserted. There are those who can assemble and combine materials for a given purpose, and there are others who can seize upon the broad consequences of a new discovery and choose the right moment for setting the old to one side. History has shown many times how the fate of a nation may depend upon its capacity to use the knowledge and the materials at its disposal. One may be reluctant to draw examples from the catastrophe of the Great War, but in its heated atmosphere

developments came quickly to maturity. During its course engines and devices of all sorts came into being which, before the war began, had never been thought of seriously, if at all. Such were tanks, paravanes, sound ranging, wireless telephony, aeroplanes, and a thousand contrivances in every section of the war on the ground, under the ground, on the sea, under the sea, in the air. Their invention and development would not have been possible if there had not been the men for the work. It was fortunate that the nation also possessed a body of young men—chemists, physicists, engineers, biologists—trained in the laboratories of universities, technical schools, polytechnics, and so on, and in industrial workshops who were able to understand and work with the new devices. I doubt if the value of those trained young men has ever been fully realized. If, unfortunately, another great war broke out, the devices of to-day would surely be modified or superseded during its course, and the process of development would begin all over again. Provided that the defence withstood the first shocks, the men with knowledge of materials and skill in using them would be in demand as before.

Though war times may furnish the more obvious examples, the developments of peace follow the same road, at a slower pace. The major industries of this country have owed their advance in part to the national resources and to political relations, but largely also to the skill of the country's scientific and technological workers. The electrical trades depend largely on discoveries which these have made and been quick to use. The same may be said of her metallurgical work, of her shipping industry, of her business in textiles, of the dye industry in which she has now taken a position which might have been hers from the beginning. The battle for the health and the nutrition of the nation depends for its success upon the same qualities. This becomes continuously more so as natural knowledge increases, and its technical use requires a more intelligent craftsmanship.

Many a similar instance might be drawn from past history. But the past differs from the present in this, that the knowledge then to be drawn upon was scantier and far less abstruse. It was related to the technical skill of the workshop rather than, as now, to the science of the laboratory. The agencies of change were such as the discovery of cast iron, the invention of printing, the design of the ocean-going chronometer, the eighteenth-century additions to the loom, and so on. To-day great matters turn upon the complicated physical science of the wireless valve or the intricacies of the internal combustion engine, or the highly-skilled chemistry that brings assistance to medicine or the combination of physics and chemistry, biology, and engineering involved in the preservation of food.

From this point of view the suggestion sometimes made that scientific workers might take a holiday looks more ridiculous than ever. No nation could afford such an intellectual disarmament in the face of the world; nor could the world itself in face of the evils that are to be overcome.

The position of the men, and especially of the younger men, who are encouraged by these financial aids to devote the most ingenious years of their life to scientific research must be considered by those to whom the ordering of their lives is due. Some of the most brilliant young men in the Empire are selected for a specific purpose, which purpose they undoubtedly fulfil. Good work is done, and when it is finished a fine and most useful type of man is available for further service. In a great number of cases the satisfactory opportunity of further service presents itself. But it is not always so. It is possible to find a man living on income derived from one Research Trust after another until he ceases from age or other limitations to be eligible for further aid. His work may have been excellent, and his competence as great as ever, but he finds that he must look in some new direction for his living. Academic activities may be no use to him, nor he to them. His occupation has led him up a blind alley. I am told that there is a certain tendency for men who have been employed in industry as research workers to change over, when possible, to purely administrative work which is expected to be more lasting and in the end more remunerative. There is here a hint as to the true cause of the trouble. The blind alley should be a thoroughfare leading to occupations more suitable to the men and better fitted to get the best out of them. It is obvious what these occupations are. They are places of responsibility to which specialists in science are as yet but rarely admitted. There is an encouraging beginning, but it takes time to realize that the man who is in touch on one side with the growth of natural knowledge should be in close touch on the other side with the opportunities of its application. He should be an equal in the council chamber rather than a subordinate in the waiting-room. On the other hand, the scientific expert must himself help to take down the barricade that makes the alley blind. This requires that his education should be much more than sufficient to make him only a laboratory man: which brings us back again to the very important point that the man himself must be as much the care of those who give him research work to do as is the work which they set before him. Obviously, the more complete the equipment of the man, the better the chance that he will make his way, and the wider his final influence. The bodies that administer research funds are already beginning to consult each other for the sake of better efficiency in the choice

and direction of workers. As this becomes more general, there will surely be an effort to take a wider view of the responsibilities which the magnificent generosity of public men has placed upon them.

Reference is made in the Report to a plan of research on malaria in India. I think that I may well amplify the reference by describing rather more fully the proposals of Council in respect to Indian Medical Research, particularly as they involve the adoption of a special policy.

In 1924 the Royal Society received a legacy of £10,000, and in 1925 £28,108 19s. 6d., being part of the residue of an estate, for the prosecution of original research in medicine, for the prevention of disease and relief of suffering, with special reference to tropical diseases in British Possessions. There are particular reasons associated with the gift for connecting its use with India. Council decided at its meeting on 9 July that the whole income, together with the invested income, shall be employed for five years (*i.e.*, until 31 October, 1941) as follows :

A.—*Malaria research*—It decided to offer to Colonel Sinton, I.M.S., a stipend for five years to enable him to work at the Horton Centre on certain aspects of malaria. The Horton Centre will be under the control of the London School of Hygiene and Tropical Medicine, and unique opportunities will be available there for clinical study, for observations on the malaria parasite in man and *Anopheles*, for investigation of the serology and immunology of malaria, and for chemo-therapeutic testing and experimentation on the human subject. Colonel Sinton is now, and has been for many years, actively engaged in malaria work in India.

B.—*Experimental studies on the ecology of certain species of Anopheles*—Where the control of malaria is successful it is nearly always achieved by measures directed against *Anopheles*. A detailed plan has been suggested by the London School of Hygiene and Tropical Medicine, by which a young man experienced in modern experimental zoology should be given opportunity for 12 to 18 months, at that School, to learn about mosquitoes in general and the Oriental species of *Anopheles* in particular, and should undertake experimental work in the physiology and behaviour of *A. maculipennis*. He should then be sent for 2½ to 3 years to an appropriate centre in India to carry out a programme connecting malaria with the behaviour of *Anopheles*. Council was informed that for the tropical portion of the scheme part of the cost could probably be met by the London School of Hygiene and Tropical Medicine. Assuming that this is the case, it decided that a sum up to £3,750 be offered provisionally, over a period of five years, to finance the project. A suitable investigator has been appointed in Mr. Muirhead Thomson.

C.—*Nutrition in India*—The existence of widespread malnutrition in India is beyond dispute, but more detailed and intimate information is required as to its incidence and effects. Council decided that Dr. C. Wilson should be offered a research grant for one year in the first instance, with possible extension for two more years, to enable her to make a survey of the nutritional condition of Indian families and to draw up a report. A study of the incidence of malnutrition among school-children, an investigation of dietary habits, an assessment of the value of foods in common use, etc., would be made in collaboration with the Indian Research Funds Association and with Dr. Aykroyd, its Director of Nutrition Research. Dr. Wilson will be able, if necessary, to work for only part of the year in India, returning to England to carry out a statistical and experimental analysis of her results. One of her objects while in India will be to build up and train a small body of collaborators by which the work will be aided and perpetuated.

The total estimated cost of the three schemes, over a period of five years, is £8,550. The Council of the Royal Society believes that by a far-reaching plan of this character, involving work of three different kinds all bearing on health in India, its Medical Research Fund can be better employed than by small grants made from time to time for worthy but minor purposes. It could make good use of far more substantial funds on analogous lines.

Reference is made in the Report of Council to the decision in the matter of the Postal Ballot; this required the invocation of a curious provision contained in our ancient Charters. We were directed, in cases of a difference which we could not settle ourselves—and in this instance our legal advisers had been unable to settle it for us—to call in the services of certain High Officers of State. This we did, and the officers in question responded promptly, taking, I believe, no little interest in this ancient direction and its present application. We are greatly in their debt for their very kind assistance.

The great increase in the amount of material to be published has brought with it certain serious inconveniences. It has always been the practice of the Society to scrutinize with great care all papers submitted to it. Fellows have been ready to undertake this task, though, as we all know by experience, the labour involved is serious. Three times as many papers have now to be examined, as compared with a few years ago, and there has been no material increase in the number of those available as referees. It is not surprising that men who lead busy lives find it difficult to attend promptly to the work which they are asked to do, especially as the intricacies of modern science may make it necessary for

a referee to devote days to any one paper. If there is much delay, there is disappointment at the tardy publication of matter which the writer naturally thinks ought to appear at once.

The Council has considered this matter carefully, and has come to the conclusion that in the great majority of cases the summary of a paper might be set up in type and distributed within a very few weeks of its receipt, without waiting for the verdict of the referees on the paper as a whole. A Fellow who communicates a paper will, of course, take the responsibility for the summary. The reading of the paper and its publication will follow in due course, as the responsible committees advise, on receipt of the opinions of the referees.

Three years ago Sir Gowland Hopkins in his Presidential Address spoke with admiration of the work of the organic chemist and in particular of the "emergence of power to grasp the architecture of complex invisible entities such as organic molecules and the ability to construct them at will". He told how under modern methods of investigation the picture which the chemist had formed of the invisible molecule had actually taken shape. His picture-making had been amply justified. His stereochemistry was not, as some thinkers had maintained, to be swept away in favour of a mathematical symbolism.

This anticipation has been fully realized during the last few years, mainly through the remarkable increase in the accuracy with which the structure of molecules, molecular aggregates, and solid bodies in general can be determined. For this the methods of X-ray analysis of crystalline structure have been largely responsible. Moreover, other methods have been greatly strengthened by the example set by X-ray analysis and by its reactions upon themselves. Optical, electrical, magnetic, and other properties have been successfully studied with the same great purpose, viz., the correlation between the properties of a substance and the spatial arrangements of its components.

While the X-ray methods have been mainly useful in describing the arrangement of the atoms in assemblages surrounded by others of like nature and conditions, the methods of electron-diffraction are giving a remarkable insight into the modifications of arrangement that are to be found on surfaces. The extraordinary interest of such knowledge arises from the fact that natural processes so largely depend on surface actions.

For many years after its inception the X-ray analysis was, as might be expected, engaged in trying its own powers and learning how to apply them. It cleared up many structural problems on which older methods had little to say that was definite, as, for example, the distinction between

ionic, metallic, adamantine, and molecular compounds. Many crystalline structures were determined, and the results, as is well known, have been serviceable in a wide field of scientific research, and in many industrial processes. The methods of analysis, the technique, and the interpretation of results have been greatly improved, as might be expected, by the researches of many hundreds of workers. The increase in accuracy is so great that new possibilities of usefulness come into view.

The improvement appears in two ways. In the first of the two, the measurements of the dimensions of the unit of pattern of a structure can now be made to one part in several thousand. Consequently, the determination of the electron charge e , made by the X-ray method, can stand beside the older determinations of the oil-drop method. There is a persistent discrepancy of about one part in two hundred, the former giving the value 4.80×10^{-10} , the latter 4.77×10^{-10} ; but it is clear that the larger value is at least as near the true value as the smaller. A full discussion of the X-ray method is given by Compton and Allison in their recent book on "X-rays", and a critical examination of some outstanding points is made by du Mond and Bollman ('Phys. Rev.', September, 1936).

Again, as has been observed by Bernal, the use of high-precision determinations of the lattice constants of metals will soon become the most reliable gauge of purity of a metallic element. Accuracy has here been pushed to one or two parts in forty thousand. Again, the phase boundaries of an alloy can be very closely and conveniently defined by observations of such a character. Accuracy has been of great importance to the well-known work of Hume Rothery on alloy structures, and to the curious and very important relations between order and disorder in alloys which have been specially studied at Manchester.

The accuracy with which the position of each atom in the unit cell can be measured is of quite a different order. Thanks in particular to the use of Fourier analyses by J. M. Robertson and others, the distances separating the atoms, centre to centre, can be found to about 1%, even when the complicated molecules of organic crystals are under examination. This is a great advance on the possibilities of even a few years ago, and it has important consequences. In particular, fresh light is thrown upon the problem of the chemical bond. At one time, single, double, and triple bonds were considered to be distinct and definite phenomena. The tetravalency of carbon, for example, was described as an assemblage of four equal powers of combination, of which one or more might be exercised in the same direction. When the diamond structure was found by the X-ray methods, it was no matter of surprise that the four separate

single bonds were displayed in the attachment of each carbon to four neighbours. In the structure assigned by the chemist to benzene, the fact that each atom had but three neighbours presented difficulties; various theories have been suggested in explanation, mostly little more than different ways of drawing diagrams, in which four single bonds were made to act somehow. In recent years, it has been more usual to propose that bonds may alternate between single and double, and that the tetravalency of carbon in the benzene ring is satisfied because three of the six links are double and three single, the two kinds alternating both in time and in order round the ring. The conception can be extended to cases much more complicated provided that the two forms between which alternation occurs do not differ much either in form or energy. The effect is described as one of "resonance", a term due to Hund but applied to organic chemistry mainly by Pauling and his collaborators. Its bearing on structural chemistry was discussed by Sidgwick a few months ago in a presidential address to the Chemical Society.

When substances in which this "resonance" is supposed to occur are examined by the X-rays, it is found that the actual centre to centre distance of two atoms connected by alternating a link between single and double is characteristic of neither of the two extremes. These last two are definite quantities, and the length of the varying link lies between them. An actual link is rarely a pure single or double or triple link. Pauling and Sidgwick both discuss a number of cases in which the centre to centre distances can be correlated with a probable or possible amount of resonance. An excellent example is furnished by oxalic acid which was examined by Zachariasen in 1934, but has just been remeasured by Robertson, using the powerful Fourier method of analysing the observations. The distance between the carbon atoms is 1.43 Å. The length of the single link of diamond is 1.54 Å. The length of a double bond is very nearly 1.33 Å. It might seem that in oxalic acid the link is actually more nearly double than single; but this is not so. A small proportion of double linking seems to shorten the distance considerably. For instance, each link in the hexagonal network of graphite must be two-thirds single and one-third double, yet its length is 1.41 Å. In benzene, the half-and-half arrangement (following Kékulé) is correlated with a length of 1.39 or 1.40 Å. Thus the actual length of a bond may prove to be a safe indication of its nature. Robertson points out that the oxalic acid molecule is always planar, which may be accounted for on the ground that rotation is restricted round a link which is even partially of a double character.

It has recently been shown by Bernal and Megaw ('Proc. Roy. Soc.,' A, vol. 151, p. 384 (1935)) that in all probability there are two types of

bond linking oxygen atoms through intermediary hydrogens. The one is the "hydrogen bond"; it is found, for example, in acids, and it corresponds to a separation distance, oxygen to oxygen, of 2.55 Å. The other is the hydroxyl bond; it is found in a number of hydroxides, and its length is about 2.8 Å. By the use of this conception it has been found possible to locate the positions of the hydrogen atoms in several hydroxide structures, particularly in the clayey mineral hydrargillite. The oxalic structure of Robertson seems to supply a new and interesting example of the difference between the two kinds of bond. One of the oxygens at each end of the oxalic acid molecule is bound to a water molecule in the crystal by a link 2.87 Å. the other by a link 2.52 Å.

It has been pointed out (Fricke, 'Koll. Z.,' vol. 69, p. 312 (1934)) that the linking up of hydroxyl bonds explains the properties of the gels that are formed by neutral hydroxides.

These few examples may serve to show how improvements in the technique of X-ray analysis are sharpening a tool which has already been of assistance to research in many directions and now seems to be acquiring a new usefulness.

The chemist has already shown that the properties of the molecule depend on the internal disposition of its atoms. The characteristics of the solid state depend also on spatial relations, and in a manner which is even more complicated, much more complicated than in the case of the independent molecule. Accurate measurement of the spatial arrangements lays a firm foundation for the study of the properties of a substance in relation to its structure and its composition. The problems to be solved are, of course, extremely complex, but it is surprising how much can be done towards the examination of intricate molecular associations when the spatial relations between the most commonly occurring atoms are known. This applies, for example, to the study of the proteins which has already gone far; to the clays, and to the glasses and other extended structures. At one time it seemed hopeless to expect to learn much of the structure of bodies which were so irregular as to give no sign of crystallinity. But it is now possible to work from the regularity in occurrence of a few definite separation distances, even when regularity in orientation does not exist: and methods have been devised by which these distances can be determined by the X-ray methods.

It is clear, I think, that the stereometry which the chemist has developed so successfully is acquiring new powers which will have the widest applications.

Awards of Medals, 1936

SIR ARTHUR EVANS is awarded the COPLEY MEDAL. He is the leading British authority in classical archaeological studies: from his father he inherited a predilection for numismatics, a subject to which, in his earlier years, he made contributions of outstanding importance. His researches in Crete from 1893 onwards resulted in the discovery of the remains of a civilization which he named Minoan after the Sea-King, Minos. He traced the development of the Minoan civilization from approximately 3200 to 1400 B.C., from the late Neolithic through the Bronze Age. His Cretan work, published in six volumes—"The Palace of Minos"—has revolutionized our knowledge of the ancient history of the Near East.

The RUMFORD MEDAL is awarded to Professor ERNEST GEORGE COKER, who has devoted a lifetime to the investigation of stresses in solids by means of polarized light. The original effect was discovered by Brewster in 1815, and Brewster himself suggested that the effect might be used for the direct exploration of stress. But although many physicists since Brewster's time investigated the effect, no one, until Coker came, developed a practical method enabling stresses in a model to be actually traced. Coker not only applied to such researches a material, celluloid, not hitherto employed and capable of being cut to any shape, but he was the first to devise a reliable instrument (the lateral extensometer) for measuring at any point the sum of the principal stresses, which is not directly given by the optical results. In many other ways he made very important improvements in methods of observation, and he has, in fact, created a new technique and given the engineer a new instrument of discovery. His work is only now beginning to be recognized and taken up by the engineer; it is not too much to say that photo-elasticity will do for engineering what the application of spectroscopy has done for astronomy; the two cases are somewhat analogous, in each a previously known physical effect has been applied to explore a whole field of new facts.

Coker's first paper on this subject dated from 1910; since then he has published, either alone or with pupils and colleagues, some 50 or 60 papers, in which he has applied the method to almost every important engineering problem which can be approximated to on two-dimensional lines. In the course of this work not only has he cleared up a vast number of problems and difficulties, but he has rendered a great service

to applied mathematics, by demonstrating to the engineer the substantial accuracy of the results of the Mathematical Theory of Elasticity.

Coker's work fulfils admirably the condition laid down for the Rumford Medal which says that the discoveries for which it is awarded should be such as "tend most to promote the good of mankind". The results obtained by photoelasticity in such subjects as the stability of dams and the strength of aeroplane frameworks will certainly be the means of saving many lives.

Moreover, Coker has created a world-wide school: to his inspiration is largely due the modern development of photo-elastic laboratories in Japan, the United States, Belgium, Switzerland, and Russia, and many of the leading workers in these laboratories have started their researches under his auspices and have remained in touch with him ever since.

A ROYAL MEDAL is awarded to Professor RALPH HOWARD FOWLER. His general theory of statistical mechanics, his later applications of it to the equilibrium of mixed crystals and to a theory of semi-conductors are of outstanding importance. His paper on dense stellar matter contains the first working out of the properties of a degenerate electron gas, and those on the internal conversion of γ -rays and on thermionic emission and stray field emission of electrons from metals were fundamental. His work on the theory of the photo-electric effect, especially as a function of temperature, led to a rational means of analysing emission frequency curves, which enables one to determine the true threshold frequencies. His work on the quantum theory of energy exchange between gases and solids broke new ground. Further, he has made notable contributions to the theory of the photo-electric current in semi-conductors, and has made notable contributions in recent years by his adaptation of wave mechanics, as it has been developed, to the solution of problems actually under investigation by groups of experimentalists working in close association with him.

A ROYAL MEDAL is awarded to Professor EDWIN STEPHEN GOODRICH. He is distinguished for his long series of researches on the Comparative Anatomy, Embryology, and Palaeontology of Invertebrates and Vertebrates, which have thrown light on some of the most fundamental problems of Animal Morphology. His work on the excretory organs of Annelids and of Amphioxus resulted in a new conception of the nephridia and their relation to the coelom and coelomoducts. His memoirs on scales of fishes and on the median and paired fins threw fresh light on the classification and phylogeny of fishes and on the evolution of the verte-

brate skeleton. His contributions to our knowledge of the segmentation of the vertebrate head are recognized as of the first importance. His volume on Cyclostomes and Fishes in Ray Lankester's "Treatise on Zoology" and his more recent book, "Studies on the Structure and Development of Vertebrates," are masterly and original contributions to the subject of comparative anatomy. All his writings are distinguished by breadth of view, clarity of thought and expression, and mastery of technical methods. In the opinion of many he is the outstanding morphologist of our time.

The DAVY MEDAL is awarded to Professor WILLIAM ARTHUR BONE, who has had a wide and varied experience in the main branches of chemical science for a period of well over forty years and has by his masterly grasp of experimental technique made many discoveries and inventions of great scientific significance.

His early work on the alkyl substituted succinic acids and allied substances, which furnished a valuable chapter in synthetic organic chemistry, was speedily followed by more fundamental researches on the thermal decomposition of hydrocarbons and their oxidation products and by quantitative studies of hydrocarbon combustion. The latter researches, which occupied him for many years, were admirably summarized and illustrated experimentally in his Bakerian lecture of 1932. The evidence collected during these prolonged and systematic investigations, devised to include a great variety of experimental conditions of slow combustion and detonation, was overwhelmingly in favour of the hydroxylation theory of the combustion of hydrocarbons.

His study of the direct union of carbon and hydrogen led to a synthesis of methane from its elements. These researches on gaseous hydrocarbons and their oxidation products necessitated accurate gas analyses, and reference should be made to his improvements in the laboratory apparatus required in such determinations.

The closely related problem of flame movements in mixtures of burning or exploding gas has been submitted by him to exhaustive examination with improved methods, which made it possible to observe for the first time some striking characteristics of this phenomenon.

On the subject of catalysis, his contributions are of especial interest and importance. Thus he has recently demonstrated by a variety of convincing experiments that carbonic oxide and oxygen will unite in the gaseous phase in the absence of moisture. At an early stage in his career Bone adopted the now almost universally accepted view of the general

nature of contact catalysis to interpret experiments on the interaction of hydrogen and oxygen.

In collaboration with many colleagues and students, Bone has investigated the difficult problem of the chemistry of coal and has applied new physical and chemical methods of identifying the constituents of this complex material. The benzenoid constitution of coals of widely different geological ages and maturity was demonstrated by oxidation experiments which furnished a complete series of the polycarboxylic acids of benzene. In this field of chemical research, which is undoubtedly of the utmost importance to all coal producing countries, Bone will always be regarded as a pioneer.

The DARWIN MEDAL is awarded to Dr. EDGAR JAMES ALLEN, who for the past forty-two years has been Director of the Laboratory of the Marine Biological Association at Plymouth. Under his wise guidance the institution has risen from small beginnings to the premier position which it holds to-day.

Allen has made many notable additions to our knowledge of marine biology, dealing with such diverse subjects as the nervous system of the lobster, faunistic studies of estuarine and other areas, systematic work on the Polychaeta, the genetics of *Gammarus*, and contributions to fishery science. He initiated exact work on the association of bottom faunas with the nature of the deposits and has published valuable papers on the artificial culture of phytoplankton, in which he was one of the earliest to obtain successful results. Many years ago he followed the chain of events leading from sunshine and inorganic constituents of sea water, through phytoplankton and zooplankton to food fishes, making some of the first contributions to a subject which has since shown most striking developments.

When the International Fishery Investigations began, Allen undertook the English share of the work, which was then based on laboratories at Plymouth and Lowestoft: after the war the Lowestoft laboratory branched off and became the Government centre for economic fisheries investigations. He has been closely associated with the International Council for the Exploration of the Sea from its earliest beginnings. The organization of the work on water pollution, carried out by the River Tees Survey Committee, owes much to his initiative, and he is a member of the Committee dealing with similar problems in the River Mersey. He received the Hansen Memorial Medal and prize at Copenhagen in 1923 and was the Linnean Medallist in 1926.

Much of Allen's work has been directed to the study of evolution.

The Hooker lecture, which he delivered to the Linnean Society in 1929, deals with the origin of adaptations, and his presidential address to Section "D" of the British Association in 1922 is again concerned with the evolution of life in the sea.

Throughout his long term of office at Plymouth, he has been the inspiration of the workers at the laboratory, and the success of his labours is shown by the high quality of the research which has been carried out under his direction. Many discoveries of fundamental importance have been made and, one after another, new methods of oceanographic research have been worked out, to be adopted at once by fishery departments and marine biologists in all parts of the world.

The HUGHES MEDAL is awarded to Dr. WALTER SCHOTTKY of the Central Laboratory of Siemens & Halske, Berlin. He is best known by his contributions to the fundamental theory of thermionic emission. One of his investigations led in 1914 to the formulation of a theory of the effect of an electric field at the surface of a hot conductor upon the emission of electrons; another led to the discovery of the "Schrot" effect, which attributes certain variations in a thermionic discharge to random emission of individual electrons. He also discovered the so-called "temperature effect" in ordinary conductors of electricity, which he traced to the thermal agitation of the molecules. Further, in the early days of the subject, he made valuable contributions to the theory of space charge in vacuum tubes.

Apart from these mathematical contributions to our knowledge of principles, Schottky has added very greatly to engineering progress in most branches of wireless telegraphy. In particular, he invented the screening grid valve which has everywhere superseded the triode valve in the high frequency amplifiers employed in receiving broadcast signals. He is also the inventor of the method of superheterodyne reception wherein the currents of intermediate frequency are amplified. These are two of the most important wireless inventions of the past twenty years.

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The Measurement of the Intensity and the Colour of the Light in Woods by Means of Emission and Rectifier Photoelectric Cells

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INTRODUCTION

For many years past the subject of plant distribution in relation to the illumination of the site has been of interest to botanists.

Results obtained using photochemical and photographic methods have been summarized by Wiesner (1907) and by Rübel (1928). Dorno (1919, 1928) has studied the measurement of daylight by a variety of methods. Klugh (1925, 1927) has given a general review of recent literature, and described a photographic method with a neutral wedge and rhodamine B paper.

Though for certain purposes photographic methods alone can be employed, these can only be regarded as simple when one is prepared to sacrifice even a very moderate degree of accuracy. The elaborate precautions necessary to obtain an accuracy of 5% or, under the best conditions, 1% have been well described by Dobson, Griffith, and Harrison (1926).

Researches upon tree growth in relation to light intensity have been carried out by G. P. Burns (1923), and G. R. Burns (1927) has described a portable radiometer. By screening the thermopile with filters, portions of the spectrum can be measured. Since a thermopile is sensitive to all wave-lengths, errors may arise through the transmission of infra-red and from radiation from the hot filters.

McLean (1919) and Klugh used a spectrophotometer. This is a valuable method, but difficulty arises in connexion with the maintenance of a source for comparison.

The measurements described give the relation between the illumination received within the wood, as a rule upon a horizontal surface, and the

illumination outside received from sun and sky. Since the illumination from the sun is very variable and that in the wood depends upon that from the sky, this basis is a shifting one.

Valuable observations were made by Abbot (1922) using an auxiliary radiometer of his astrophysical outfit in a grove of beech and black birch on Hump Mt., N. Carolina. As these measurements have been overlooked by botanists they are summarized here. Five sites were examined: (a) In shade of two large trees, no undergrowth within several feet. (b) Under some rather thick saplings, partly shaded by higher trees, thin undergrowth. (c) In a bed of ferns, which covered the instrument, but with an open space directly above. (d) In open space; trees all around, ferns and other vegetation somewhat above instruments towards south. (e) Thick growth of saplings, considerable small vegetation on ground.

The measurements in the open were estimated from known values. On 7 September, 1917, the sky was overcast, so the results are true "radiation factors", as defined later. On 12 September, there was a cloudless sky. On 2 October the positions were the same, the sky cloudless and very clear. The leaves were beginning to fall and many had turned yellow. The percentage illuminations at the sites *a*, *b*, *c*, *d*, and *e* were respectively 3.6, 3.8, 4.1, 16.3, and 3.8 on 7 September; these are quite comparable with some of our results. The percentages are smaller when the outside illumination includes sunlight as on 12 September, when they were 2.2, 1.5, 1.1, 3.9, and 2.1%. On 2 October the corresponding values were 2.3, 1.5, 0.9, —, and 2.3%.

For our work photoelectric cells were selected (1926, 1929, 1930, 1931) as having many advantages, though we also used a photochemical method with uranyl oxalate (1929) which gives a useful time integration.

When a photoelectric cell is exposed to light and connected, the readings obtained are a measure of the electric current generated by the energy falling on the sensitive surface of the cell. The measurement does not give the illumination, unless carried out after a careful examination of the properties of the cell and with due regard to the optical conditions under which the light reaches it.

OPTICAL CONSIDERATIONS

The total quantity of light, regardless of angle, is what appears to be most important for plant growth. This may be called the Total Illumination, *I*, and may be defined as follows. Let $i/d\Omega$ be the illumination in

lux due to a small pencil of rays of solid angle $d\Omega$ on a small area placed perpendicular to the pencil. Then

$$I = \int_0^{4\pi} i d\Omega.$$

This measures the total illumination that is available for photosynthesis at a given point. In practice one can replace the ideal point by a small sphere. We previously suggested the use of a sphere filled with uranyl oxalate and more recently (1936, *a*) we constructed an opal glass globe, with additional internal diffusing surfaces above a photocell, so as to measure the light incident on the spherical surface irrespective of altitude and azimuth. But we have not as yet used either method for the study of illumination in woods, though uranyl oxalate tubes were so used.

One may also define the illumination as that received upon a plane surface set either vertically in some stated azimuth, or horizontally, or in some intermediate position so as to catch the maximum amount of light from sun and sky; these may be termed, respectively, the horizontal, vertical, and maximum illuminations and denoted by the letters H, V, and M. The horizontal component H, received on a vertical surface, is of importance where tubes containing a photosensitive liquid are exposed. In general, however, our practice has been to measure V, as received upon a horizontal surface. Sometimes, as near the edge of a wood, the maximum illumination is what governs plant distribution, for the leaves may be set so as to receive the best illumination possible; accordingly, in such sites M has often been measured.

The nature of the receiving surface of the measuring instrument also requires consideration. We finally selected opal flashed glass as giving complete diffusion and being permanent in its properties. For use in air a matt surface is desirable, though probably not perfectly constant and obviously affected by water drops. As our outfit was originally intended for use at sea, we adopted, for all purposes, a smooth flashed surface. This has involved the necessity of correcting for reflexion losses. The diffused light from a uniformly illuminated sky may be represented by a single beam at 30° altitude. For this the reflexion loss factor is 1.06 (Table I, 1926), but at 35° the factor is only 1.03, and at 40° it is 1.02. When dealing with light from sun and sky separately, we have used the value 1.06 for sky and the factor appropriate for the altitude of the sun. The effects have been distinguished by screening off the sun's rays by the interposition of a small object held at about a metre from the photometer. The ratio of illumination from sun and sky to sky alone may be denoted by β ; accordingly the ratio of sun to sky is $\beta - 1$. When β has not been

determined we have used the diffuse light factor, 1.06, in measuring the general illumination; this tends to give values which are rather too great when α , the sun's altitude, is great, and rather underrates the illumination for low angle sunlight. As the changes in illumination throughout the day are so great and their variation is so rapid the reflexion loss errors are not serious.

The reasons for using a diffusing surface over the photo-cell should be clearly appreciated. When illuminated, this acts as a secondary source of radiation for the photo-cell; this is at a constant distance with constant angular distribution; all that one measures is accordingly the variations in its brightness. The diffusing surface of the glass is mounted away from the cell, so that, when standardizing, it can be set at an exact distance from the primary source. (For details *see* our 1935 paper.) The opalized surface (opal) is mounted flush with the rim of the photometer, to avoid screening of low angle sky light; such screening occurs to some extent with the plane surface rectifier cells; but with emission cells it is always serious. Cells mounted for immersion in water are specially subject to this screening error. A cell thus mounted was used without an opal by Carter (1934) in woods; it accordingly gave erroneous readings. There is, furthermore, the serious error due to an unknown and variable angle of incidence for undiffused light reaching the curved surfaces of many types of emission cells. Thus no accurate use of these cells is possible, save for measurements with light incident normally, unless a diffusing surface is interposed. The opal has the disadvantage, which is usually quite unimportant, of reducing the reading to about 45%, but where the rim effect is important, an opal interposed may actually raise the reading. It also has some selective action, since its transmission is appreciably higher in the red than in the blue, especially with the opalized surface on glass tinted slightly green. In practice this selective absorption is unimportant.

PHOTOELECTRIC CELLS USED AND METHODS OF MEASURING THE PHOTOELECTRIC CURRENT

In our earliest work (1926) we used small vacuum emission cells with an unsensitized potassium film sensitive only to the blue end of the spectrum; as a measuring instrument the potentiometer-telephone-amplifier outfit (Poole, 1925) devised for work at sea gave adequate sensitivity and permitted of the study of the coastal wood at Cawsand on Plymouth Sound. Its weight was its drawback for field work.

The advent of the large and highly sensitive Burt vacuum sodium cells enabled us to go farther afield, using the very sensitive Onwood type of pointer microammeter made by Gambrell (1929, 1930). Its special type of suspension gives the most sensitive instrument we have found for field work. It will function when put down on any approximately level spot, and its full scale, $12\ \mu\text{a}$, is subdivided to read $0.1\ \mu\text{a}$ and may be estimated to $0.02\ \mu\text{a}$. The resistance is about 300 ohms.

To measure the radiation throughout the visible spectrum, we used the thin film caesium cell of the General Electric Co., type C.M.V. 6, with filters (1931). For the combination the microammeter was of inadequate sensitivity in heavy shade. A more sensitive portable Onwood galvanometer was accordingly mounted in a box with scale at one metre. The sensitivity, 2.89×10^{-9} amp. per 1.0 mm. deflexion was adequate. Measurements were made with the galvanometer in a building and the cells in the open, under trees; but in the open, vibration due to the wind caused trouble. The bulk of the measurements were accordingly made by means of J. H. J. Poole's neon tube photometer (1928). This gave sufficient sensitivity and was readily portable. In damp weather leakage was troublesome, though this was much reduced in a later model (Gall and Atkins, 1931).

An entirely new type of photo-cell held out hopes of the development of a lighter apparatus with adequate sensitivity. Preliminary work with a cuprous oxide rectifier (or sperrschicht) cell was carried out in a Dartmoor wood in the autumn of 1932; this showed that the cell worked well in spite of the fact that it rained heavily all day; under such conditions no emission cell could be used on account of the high—and consequently variable—electrical leakage. We accordingly examined (1933) a number of these cells together with the still newer selenium rectifier cell, which has many advantages over the cuprous oxide type. Firstly, the latter are unstable and lose much of their sensitivity in a few months, whereas the former appear to be quite stable; one particular selenium cell of the Bergmann type (1931), made by the Weston Electrical Instrument Co., has remained constant, under constant conditions, probably to within 0.5%, for about a year and a half, during which it has had considerable exposure to full sunlight. Secondly, while the cuprous oxide cells examined have sensitivity maxima at 480–510 $\text{m}\mu$ and show but little response beyond 600 $\text{m}\mu$, the selenium cells have a maximum at about 590 $\text{m}\mu$ and are sensitive to the extremes of the visible spectrum and slightly beyond. Both types show curvature in their illumination—current characteristic. In some of the newer German cells of high sensitivity this curvature is so great that they are unsuited for the measure-

ment of daylight unless the intensity of the latter is greatly reduced. The Weston cells are suitable for use in full daylight. All rectifier cells show a lesser curvature when used with a low resistance measuring instrument, galvanometers having a resistance of 5 or 10 ohms being the best for work in bright daylight. For currents up to $12\text{ }\mu\text{a}$, one may use the Onwood microammeter of 300 ohms resistance. Even more important than the curvature, which can be determined once for all, is the temperature error, very complex in its causes, which is far more serious with high resistance galvanometers. This effect depends upon the temperature of the cell itself, which cannot readily be ascertained.

We have designed a shunt (fig. 1, 1933), which when used with a 10-ohm microammeter reduces the current to 0.2, 0.1, or 0.02, while the total effective resistance of the shunt with galvanometer is maintained at 10 ohms throughout. The microammeter used reads up to $240\text{ }\mu\text{a}$, so the total current measured may be up to 12 mA , which is adequate for the brightest natural illumination. For approximate measurements in the open a pocket microammeter may be used. This reads up to $500\text{ }\mu\text{a}$ in $10\text{ }\mu\text{a}$ steps; its resistance is 69 ohms and a reduction in current may be obtained, so as to keep on the scale, by means of one or more disks of solid (not flashed) opal glass, the transmissions of which, both singly and in pairs, have been previously determined. This 69 ohm galvanometer was not used in the work described here. The best way of using a rectifier cell is to have a measuring system of zero effective resistance according to the circuit described by Campbell and Freeth (1934). This, however, does not lend itself to use in the field.

The emission cells used were shown to give a rectilinear relationship between current and illumination. For the Weston selenium cells, using a 10 ohm microammeter, the relationship is given by a curve which can be expressed as $S = S_0(1 - k\sqrt{c})$, where S and S_0 are the sensitivities in microamperes per lux for a cell current of c microamperes and for very small currents, respectively, and where k is a curvature constant peculiar to each cell. For the cell we used most $k = 0.0027$; for another one $k = 0.0029$.

A convenient way of obtaining the illumination from the observed current c in microamperes is to write $f = 1 - k\sqrt{c}$ and to make a table of f for values of c increasing as the squares of the natural numbers up to 90, namely $8100\text{ }\mu\text{a}$. Then $c/f = c'$, the current corrected for curvature. This, multiplied by the reciprocal of the sensitivity of the cell, gives the illumination. With $k = 0.0027$ and a low intensity giving $c = 16\text{ }\mu\text{a}$, f is 0.989, so that neglect of curvature would only give an error of about 1%. In bright light, however, with $c = 2500\text{ }\mu\text{a}$, f is

0.865, so a serious error would be introduced by its neglect. Other Weston cells with higher values of k give an error of up to 40% in bright light if curvature be neglected.

For most ecological work sufficient accuracy is given by tabulating the sensitivity of the cell against the current between given limits. Thus one cell sent to the Malay States was shown as 16.8 lux per μa for currents up to 130 μa , 17.0 from 130–200 μa , ..., up to 27.5 for 3800–4000 μa .

The sensitivities of the cells were determined (1935) by means of a sub-standard uniplane filament vacuum tungsten lamp run at 2360° K. and a Davis Gibson filter cell containing a solution which converts the spectral character of the light from the lamp into artificial mean noon sunlight.

Table I shows how greatly the sensitivities of the photoelectric cells available have increased since 1924; thus the latest type of cell is about 200,000 times as sensitive as the earliest we used.

TABLE I

Constants (*i.e.*, reciprocals of sensitivities) of photocells, *as mounted* and under opal filters in metre candles (lux) per microampere, when standardized in normally incident artificial mean noon sunlight. Figures marked with asterisk were obtained by comparison in daylight with another cell standardized as described. The emission cells were all of the vacuum type. The aperture given is that of the diffusing window, behind which the cell is mounted.

Type of cell	Mark	Aperture diameter cm.	Lux per μa	When first used
Potassium	G	2.4	318,000*	1924
"	H	1.2	47,600	1925
Sodium	B188	10.0	6320	1928
"	B223	9.9	1370	1928
C.M.V. 6	—	3.6 × 2.4	1410	1930
X 16	498	9.0	40	1933
Cuprous oxide	A	2.0	170*	1932
Selenium	W21104/2	4.0	16.4	1933
"	E58/2	5.8	1.32*	1935

The selenium cell has appreciable sensitivity in the red, but for colour measurements in the deep red, the C.M.V. 6 cell would have been used again for studying this region were it not that the type X cell of Bolton King is more sensitive. This is a vacuum emission cell used at 60 volts anode potential. The wave-length response curves for the cells mentioned are given in fig. 1, in which they are shown not for an equal-energy spec-

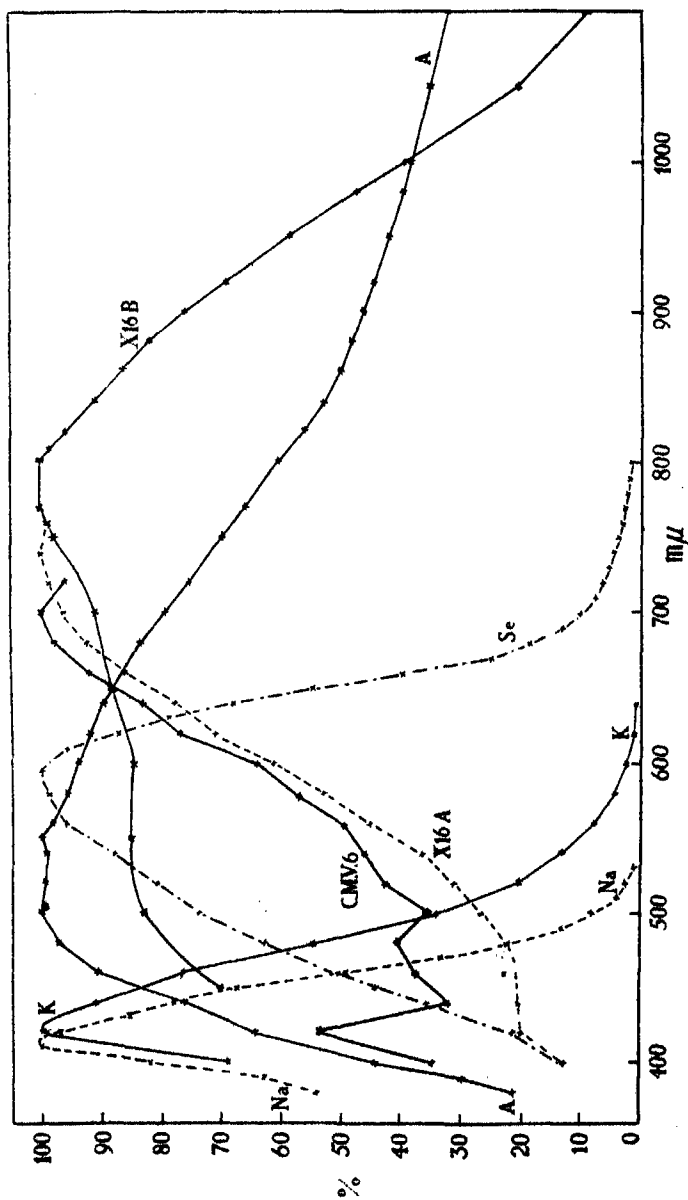


Fig. 1—Wave-length sensitivity curves for sodium, Na, potassium, K, thin film caesium, C.M.V. 6, selenium, Se, and type X 16 cells, plotted for light of the spectral energy distribution of Abbot's mean noon sunshine as shown in curve A.

trum, but after conversion to the natural "mean noon sunlight" spectrum derived from the work of Abbot quoted in the Report of the Seventh International Congress of Photography, London (1929), and extended into the infra-red from the data of Table 767, Smithsonian Physical Tables, Washington (1933), for air mass = 2. This conversion results in the curves for sodium and potassium having their maxima close together in the blue end of the spectrum, whereas in the equal-energy spectrum sodium has its maximum in the ultra-violet around 360 m μ . The curve of the Weston selenium rectifier cell is but little shifted by the change. The maxima of the C.M.V. 6 and X 16 cells are both drawn in somewhat towards the shorter wave-length since their equal-energy spectrum maxima lie in a region where the natural mean noon sunlight curve is falling. Our measurements with type X 16 were made with No. 435; another curve is also given, derived from measurements kindly supplied by the Oxford Instrument Co. This is shown because our determinations did not extend into the infra-red where the maximum is situated, but it is clear that the two cells differed considerably in the visual range. The curve for the cuprous oxide cell has been omitted since it is now of no practical importance, but the equal-energy form of this curve is given in our fig. 4 (1933).

THE ILLUMINATION DUE TO DIRECT SUNLIGHT AND TO DIFFUSE SKY LIGHT AND ITS VARIATION

The illumination in the open has already (1936, *a*) been shown to give mean values for the daily maximum of the vertical component ranging from 18.1 kilolux in December to 150.9 in July at Plymouth, as measured on the potassium cell carbon arc scale, which is very similar to the visual scale. On account of the unequal lengths of the days in the different seasons, the effect of the brightness of the five months April to August is increased. It was found that during July, 1930, at Plymouth, the daily mean value was 781.4 kilolux-hours, whereas in December it was only 82.1. Thus December received only 1.68% of the year's light, whereas July received 16.03%. During the six leafy months, May to October, 74% of the year's light is received, or if one substitute April, when the leaves are forming, for October, when they are falling, the value becomes 83%. Of the whole, a very large proportion is sky light. Meteorological returns for England, S.W., show an average of 36% of the total possible sunshine from May–October inclusive, and these include the sunniest months. In considering the effect of sunshine, it must be borne in mind that it is only operative for one-third as long as is diffuse light. It would

accordingly require to be three times as intense, as received upon a horizontal surface, to equal the effect of sky light in this climate.

Since the sky looks blue it is obviously richer in blue and other short wave radiation than it is in red and infra-red. Tables IX and X (1931) and Table VI (1936) give the results of measurements of β and sun/sky ratio, $\beta - 1$, in various spectral regions. In the blue, sunlight is often twice and but rarely three times as intense as sky light, whereas in the red the ratio may be from 5 to 6.5. These, however, are very nearly maximum values for this latitude, and in general the ratio is considerably lower.

It is thus clear that in any year the amount of blue light received on a horizontal surface from the sky must be far greater than that received from direct sunlight; also it seems likely that sky light is more important as regards red light, though probably not so for infra-red.

ILLUMINATION IN SHADED SITUATIONS AND THE DAYLIGHT FACTOR

The work of Paterson and Walsh (1915) on the illumination of rooms has a direct bearing on the lighting inside a wood. On a dull day, with a grey sky, the illumination at a point inside a building bears a very nearly constant ratio to the external illumination. This ratio, expressed as a percentage, has been termed the daylight factor, and is undoubtedly the most valuable measure of the illumination in a wood.

In a wood the illumination depends upon the brightness of the sky alone; this is because light is received from innumerable shafts of sky light, and only in a sun fleck is the intensity of sunlight of any importance. Thus on 10 September at 11.55 G.M.T. the illumination in the open, V , was 81.5 kilolux and in a wood V_w was 4.77 kl., giving V_w/V 5.8%; the sun was out with light fleecy clouds in the blue sky and $\beta = 3.1$. At 11.59 a small cloud was over the sun and V was 25.4 kl., with V_w 4.63; β was, of course, unity and $V_w/V = 18.3\%$. At 12.2, the sun was out again giving $V = 76.7$ and $V_w = 5.31$ kl. with $\beta = 3.0$, and $V_w/V = 6.9\%$. If instead of considering V_w/V , which gave 5.8, 18.3, and 6.9%, we calculate the ratio of V_w to the diffuse light in the open, namely $V_w\beta/V$, we get 18.1, 18.3, and 20.7 for the daylight factor, δ . The small rise in δ was evidently due to the bright clouds near the sun being in a favourable position for illuminating the site of the photometer. It is obvious that δ will not remain quite constant. Table II shows the variation one may expect. The shaded site was a garden with trees and shrubs, on a north aspect; the open site was the top of a hill. The site was examined on 30 May using uranyl oxalate tubes set horizontally, which

gave integrated values over three-hour periods. On 13 October we used a vacuum potassium cell which is blue sensitive, like the uranyl solution.

TABLE II

DEGREE OF CONSTANCY OF DAYLIGHT FACTOR, $\delta = \frac{V_w}{V} \beta$

G.M.T.	Notes	δ	G.M.T.	Notes	δ
5.40-8.40 a.m.	Uranyl method— Overcast morning ..	57.8	2.21	Sky clearing at zenith	61.4
8.40-11.40 a.m.	Murky then rain and clearing up. Sun obscured through- out	61.4	2.24	„ „	61.4
			2.25	Zenithal blue patches	67.7
p.m.	Potassium cell—		2.26	Bright clouds near sun	66.2
1.15	Sun out, $\beta = 1.92$	55.8	2.34	Sun still invisible ..	63.7
2.19	Uniformly overcast	56.0			
2.20	„	57.0			

In spite of some variation in the daylight factor, it is none the less, especially if averaged over a day, the best indication of the ecological lighting conditions, save at the edge of a wood, where the distribution of the vegetation may be more in agreement with $\delta_M = M_w/V$.

It remains to consider how far sun-flecks alter the total amount of light received during the day. In this climate the sun only shines for about one-third of the day. If a woodland floor has 25% of its area covered with sun-flecks when the sun is high, the amount will usually be much less with a low sun; though some areas will never receive sunlight, any given portion of the ground will receive some light from the sun for about one-fourth of the time. This is obviously a major estimate, since with low sun the area of sun flecks is smaller and might perhaps be taken as only one-quarter as much. As the sun only shines one hour in three, the sunlight effect thus becomes reduced to one-twelfth of the time, as a maximum, taking a 25% area of incidence, down to probably one forty-eighth on an average. Measurements made in Brisworthy Wood on 6 September, just before 3 p.m., G.M.T., showed that close to deep shade, where the selenium cell under opal gave $\delta = 3.9\%$, the illumination in a sun fleck was two to five times as great as in the shade. The average illumination might therefore be somewhere near $\delta + 28/48$ to $\delta + 58/48$. Such values are sufficiently close to δ not to invalidate it as

an ecological measure of the lighting conditions. In a small clearing, with $\delta = 15\%$, the full intensity of the direct sunlight might be received, but this would only last for a short time, so that its effect would not be very marked, and δ would still be a useful index. The best way of dealing with such situations is obviously an integration method, such as the exposure of a photosensitive liquid with a horizontal surface.

We have thus far spoken of the illumination in a wood as if it were derived, in the absence of sun flecks, from diffuse sky light reaching the site directly through openings in the foliage, and thus undergoing no spectral change. In fairly open sites this is the main source, but there are two others: light transmitted through the leaves, and light reflected from the leaves and woody structures. These become relatively more important according as δ diminishes, and so they cause the spectral quality of the illumination to differ very considerably from that of sky light. Accordingly we can no longer speak of the daylight factor as if it were indivisible, but should specify with it the portion of the spectrum to which it relates (*see* Table IV). We have no method of distinguishing between the transmitted and the reflected light.

COLOUR FILTERS AND COLOUR FACTORS

To measure the intensities in different portions of the spectrum it is not usually possible to find a photoelectric cell which is sensitive to the desired range only. Filters having a selective action must therefore be used; glass filters are most convenient for work in the open since they are relatively unaffected by water and by intense light. Unlike liquid filters, neither glass nor gelatine can be exactly reproduced.

Difficulties are encountered in obtaining pure spectral regions; blue is usually contaminated by transmission in the deep red to which the eye is only moderately sensitive, though certain photo-cells are highly sensitive.

Most green filters also let a certain amount of red through, whilst some have a very considerable red transmission.

We now use the Jena glasses with but few exceptions, and, in some cases, work by difference between the transmissions of the filters. The data supplied by the makers show that these glasses have closely the same high infra-red transmission out to 3000 $m\mu$.

In Table V we give some of the filters used by us together with the ranges they measure. The thicknesses shown for the filters are not the distances the light travels, since they are used under the opal diffusing glass, which we have found increases their effective thickness by 15%.

The use of the opal is essential in order that the light may always traverse the same thickness of colour filter regardless of its angle of incidence. The work of Carter (1934) is vitiated by the omission of an opal.

The filters listed cover the spectrum well and by subtraction the effects due to quite short bands can be obtained by means of other filters of the GG, OG, and RG series. In view of the errors inherent in working by differences with an unsteady light source, variable also in colour, we have confined ourselves to the use of certain rather wide spectral bands as a rule. But under specially steady lighting conditions we have at times used rather narrower bands. By the aid of colour filters used with appropriate cells we can now determine the daylight factor throughout the spectrum. If the light were transmitted unaltered, namely if it all passed through the spaces between the leaves, it is obvious that the values of δ would be the same throughout the spectrum. If some penetrate through the leaves we would expect a change in its spectral composition, and this is what we find; examples are given in a later section. As a rule the red is transmitted more freely than the blue, and this may be expressed by taking δ for the blue, which is most heavily absorbed, and dividing it into δ for the red; this red/blue ratio may be termed the "colour factor" for the site.

One cannot easily obtain two photoelectric cells having exactly the same spectral sensitivity, so in order to use the cells for daylight factor determinations with filters it would be necessary to compare them in the open under each filter. Slight differences in the filters are also allowed for by this method. When the colour of the light—though not necessarily the intensity—remains constant, we may adopt a simpler and quicker method of working, and this was our usual procedure; determinations were made of the daylight factor using either a blue-sensitive cell or a cell without colour filter and the colour of the light at each site was studied, using the same cell and the same filters both outside and inside the wood.

In the open, the light is never absolutely constant, so determinations of the transmission of filters should be made by comparing each in turn with one, taken as standard, or by comparing each filter in turn with the opal only. Such comparisons were usually made against BG 12, or against RG 1 when type X 16 cell was used.

If we consider, for simplicity, the blue, green, and red, we get percentage transmissions which depend upon the filter, the source, and the cell. Taking one filter as standard, say blue, the ratios of the percentage transmissions green/blue and red/blue may be termed the "Colour Ratios" for that source, provided the same cell is used always. They

will be independent of the intensity of the source provided this alters without alteration of spectral distribution. If now we determine the same ratios with another source and take the first source as standard we obtain the "colour factors", by dividing the ratios; these are largely independent of the type of cell* used and, of course, of the intensities. We have already given examples of such determinations (1931). We now give further comparisons of the colour composition of the light in woods with that of the light of the sky. If desired, the transmissions need not be determined and the ratios green/blue and red/blue, etc., may be got by direct comparison.

MEASUREMENTS OF DAYLIGHT FACTORS AND COLOUR FACTORS IN SHADED SITUATIONS

This work has been in progress for a number of years and the equipment used at various times is described below. A certain degree of finality appears now to have been reached.

Brisworthy Wood, Dartmoor, was selected as a site for further work because it is largely composed of *Fagus sylvatica*, and we had not studied a beech wood; it contains a few trees of *Larix europaea* and *Quercus robur*. The wood is several hundred metres long on each side, and is roughly rectangular; the ground slopes slightly downwards towards the south-west. The floor of the wood is strikingly clear of undergrowth, but is carpeted in places with the moss *Polytrichum*, with dead leaves, and with grass towards the edges.

a—Using a Cuprous Oxide Rectifier Cell

The cell was sensitive to blue, green, and orange, but attempts to work with colour filters were unsuccessful on account of inadequate sensitivity in the low illumination of the wood. Table III shows the daylight factors obtained. The low values of δ account for the freedom of the wood from undergrowth, though the fact that Dartmoor ponies shelter there at times may be a contributory factor. The wood adjoins unenclosed moor, with heather and gorse on two sides.

b—Using a Selenium Rectifier Cell and Corning Filters

Almost simultaneous measurements were made, outside and inside the wood, on 4 September, 1933, using the newly introduced selenium

* This would not be strictly the case since our colours are not monochromatic and the sensitivity curve of the cell may vary within the colour band, but in general it is sufficiently exact for our purpose.

TABLE III
Daylight Factors in Brisworthy Wood

Date	Photometer	Conditions	Site	
29 August, 1932	Cuprous oxide ..	Heavy rain	Under branches of large <i>Fagus sylvatica</i>	8
	"	"	Near trunks of <i>Fagus sylvatica</i>	5.0
	"	"	Clearing inside fence, north side	3.6, 2.1, 3.5
4 September, 1933	Selenium	Cloudless blue sky	On thin grass, under slender <i>Quercus robur</i>	48
	"	"	Under large <i>F. sylvatica</i> , ground bare ..	16, 13.3*
	"	"	Opening in centre of wood, very little grass†	3, 2*
10 July, 1934	Solarimeter	Blue sky and clouds	Under branches of <i>F. sylvatica</i> on dead leaves	12
6 September, 1934	Selenium	Sun through light cloud	Near trunk of large <i>F. sylvatica</i> on moss	6.4‡
				3.9

* With blue filter.

† Also small plants of *Rubus fruticosus*, one in flower, some *Oxalis acetosella* and *Vaccinium myrtillus*.

‡ Radiation factor.

rectifier cell. The daylight factor was determined at several sites, *see* Table III.

Using as colour filters Corning blue, green, yellow, and red, and a Schott RG 5 for deep red, the colour factors were also determined. These were the filters used before with type C.M.V. 6 cell, and their transmissions are shown in fig. 1 and Table I (1931). The blue lets through a little red, but in a region in which the selenium cell is not very sensitive, so that the error is not serious.

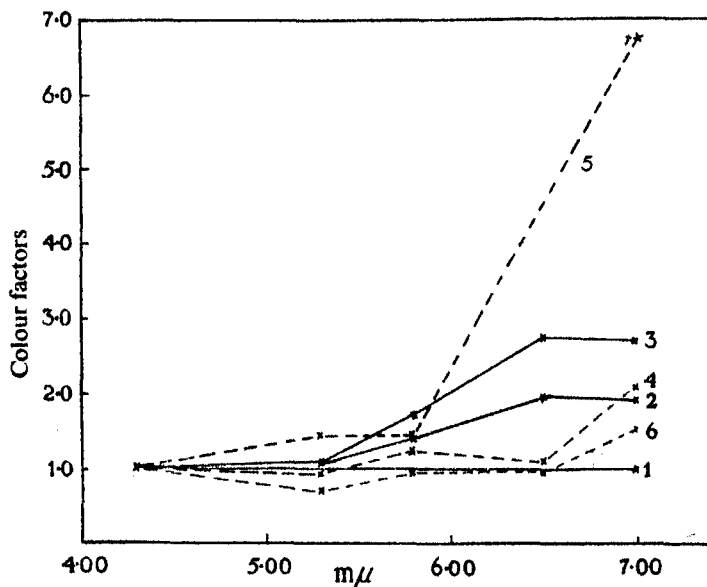


FIG. 2—To show the colour composition of the light in and outside Brisworthy Wood at 3.0–4.30 G.M.T., on afternoon of 4 September, 1933, No. 1, taken as unity for each colour, represents light from a cloudless blue sky; No. 2 shows sun and sky; No. 3 sun alone for 34° altitude. No. 4 represents light in the wood where daylight factor (for whole spectrum) was 16%; No. 5, the same, with $\delta = 3\%$; No. 6, with $\delta = 12\%$.

The results of the colour filter measurements are shown in fig. 2; the values for yellow were got by subtracting the readings for red from those for yellow, 590 mμ; similarly the deep red was subtracted from the red to get the value for 640 mμ.

c—Using Colour Filters with Selenium and Type X Cells and also Solarimeter

Measurements were made at Brisworthy Wood on 10 July, 1934, with the Moll solarimeter; changes in the radiation in the open were followed, while at work in the wood, by frequent readings of the solarimeter. When

the first set of colour measurements was being made with the cell in the open the radiation was 69.4 milliwatts per cm.² and during the last the radiation was 25.4. A thick filter of Chance's blue glass was used to eliminate all red; Chance's yellow, Corning green, and the Jena RG 1 were also used with the selenium cell.

For the deep red and infra-red the Oxford Instrument Co. type X 16 cell was used under Jena filter RG 5. This cuts off sharply at 675 m μ .

One site was examined in the wood, together with the outside site, before and after. At the start, 3-4 p.m. G.M.T., there was more direct sunlight than at the end, 5.50-6.15 p.m., yet the colour of the light had altered but little. Taking the light at the start as a basis, the light at the end was blue 1.00 (by definition), green 0.985, red 0.957; the constancy found was quite surprising. The absolute intensities had, of course, decreased greatly.

At the site, under a large beech tree, on dead leaves, the radiation factor was 6.4%. Based on the diffuse light of the sky, the colour factors for the site were blue 1.00, green 1.21, red 1.43.

When compared in the open with variable sunlight, the selenium and X 16 cells gave good accord over a wide range; thus with illuminations, by the selenium cell, 50.3, 93.3, and 106.8 kl. the X 16 cell gave 0.381, 0.389, and 0.387 kl. per μ a respectively. In heavy shade the illumination 1.105 kl. corresponded to an increased sensitivity, 0.205 kl. per μ a, since this light is relatively richer in the deep red which leaves transmit well and to which this cell is specially sensitive. Later on in the open, with less intense sunlight, namely with a bluer light, the cell gave 0.525 kl. per μ a; based on this weak sunlight, which is practically diffuse light, the deep red with infra-red was relatively 2.56 times as intense in the wood as it was in the open.

d—Using Sodium, Selenium, and Type X Cells with Jena Colour Filters

Measurements were made on 6 September, 1934, using the selenium cell and X 16 with the complete set of Jena filters and a Corning sextant green; a more detailed examination of the colour composition could thus be carried out. Measurements in the ultra-violet were also made with a Burt sodium cell and Corning red purple Corex A filter.

The daylight factors are shown in Table III. For X 16 used with the filters RG 7, 8, 5, 2, 1, and OG 2, the results are shown in fig. 3. The sensitivity with Corning green was too low for use in the wood, and with a highly red sensitive cell such as X 16 the blue filter BG 12 gives erroneous results owing to its parasitic red transmission. The comparison was

here made relative to daylight, including weak sunlight, outside. The high transmission of the leaves for the deep red and their absorption in the near red and orange is indicated. Fig. 3 also shows similar results obtained in a holm oak wood at Antony on 2 August, 1935. Owing to its high sensitivity in the infra-red, it is better to retain X 16 for measuring this region and the far red and to use the selenium cell for visible daylight.

The measurements of ultra-violet indicated that it was absorbed by the leaves rather more heavily than was the blue.

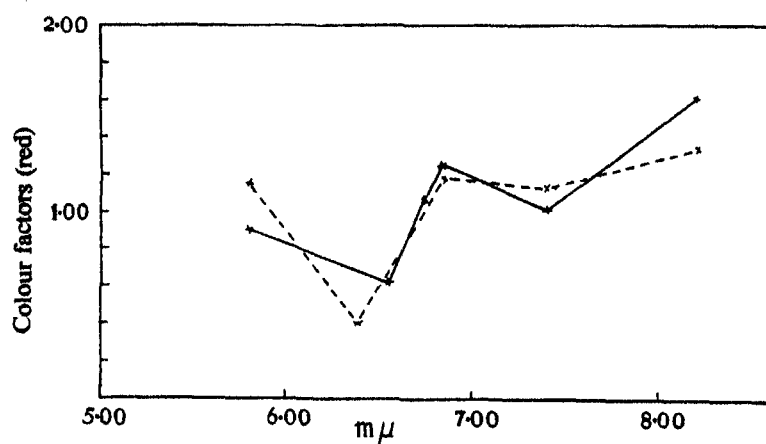


FIG. 3—Colour factors for light in Brisworthy Wood, relative to mixed weak sunshine outside on 6 September, 1934, also, dotted line, for light in holm oak wood at Antony on 2 August, 1935, relative to grey sky. Both were determined with cell X 16 and RG 1 filter as unity, viz., based on red light.

e—Final Arrangement, as in (d) but with Tapping Key in Circuit

A modified procedure was adopted for the work at Brisworthy on 1 August, 1935. A selenium cell outside was connected with the microammeter inside the wood; a similar selenium cell was placed at the site chosen for examination. It was so arranged, by means of a tapping key, that the cell in the wood was always connected to the galvanometer unless the key were pressed down; this reduces the risk of passing an unshunted current, and measurements are made more quickly than by the throw-over switch. The latter makes very good contacts, and it is essential that the tapping key contacts should also be good since one is dealing with a circuit of fairly low resistance. The results obtained are shown in Table IV. The daylight and colour factors with the deep red filters are somewhat too high, for it was found afterwards that the cell

used in the wood was rather more sensitive to this region than was the similar cell used outside. The error is only small for the red as a whole, as found using RG 1. With it, Table IV shows a colour factor 1.67, whereas a separate determination in the wood, carried out with the cell formerly used outside, gave 1.60.

TABLE IV

Brisworthy Wood, 1 August, 1935, a calm, sunless afternoon. Daylight factors with various colour filters, and corresponding colour factors, in wood on moss and dead leaves under beech tree 22 m. in from S.W. corner of wood.

Illumination in open 24 kl. at start, radiation $18.1\text{--}21.7$ mw. cm.^{-2} throughout.

Filter	Daylight factor %	Colour factor
Opal alone	2.68	1.25
BG 12	2.14	1.00
Corning green	2.38	1.11
GG 3	2.30	1.07
GG 11	2.89	1.35
OG 2	3.30	1.54
RG 1	3.57	1.67
RG 5	7.54*	3.52*
RG 8	12.3*	5.76*

* Too high, see text.

To avoid this error, the same cell was used for determining colour ratios outside the wood and inside where the daylight factor for cell with opal was 2.68%. Here the solarimeter gave $\delta = 5.4\%$, the radiation being 0.94 mw. cm.^{-2} in the wood at the time. A site farther in was also examined, under a beech tree with large beech trees and *Pinus sylvestris* around. Here $\delta = 2.0$ using the selenium cell. Fig. 4 shows the colour factors at these two sites, also a set obtained similarly on 2 August in a wood near Antony, Cornwall, under *Quercus ilex*, on ground carpeted with *Hedera helix*. As on 1st, there was a grey sky, with no shadows visible in the open. It was very windy, so measurements in a wood were less accurate on account of the swaying of the trees which causes the light below to vary considerably; even in the lulls about 3–9% variation was observed; mean values were taken. The daylight factor, as found with selenium cell and opal only, was almost identical with the Brisworthy Wood site of the previous day; successive determinations gave $\delta = 2.69$ and 2.64% at the start and 2.46 later on, values which illustrate the

constancy of the factor in time. The solarimeter gave $\delta = 5.4\%$, the same as at Brisworthy. Thus in both woods, for $\delta = 2.68$ with selenium cell, the total radiation value of δ was 5.4, viz., just over twice as great. This is additional evidence of the highly selective character of the absorption due to the foliage. The dark green foliage of *Q. ilex* gives a curve which is decidedly different from that of the beech wood.

Fig. 5 shows the daylight factors for the two sites in Brisworthy Wood examined on 1 August, 1935, plotted against wave-length.

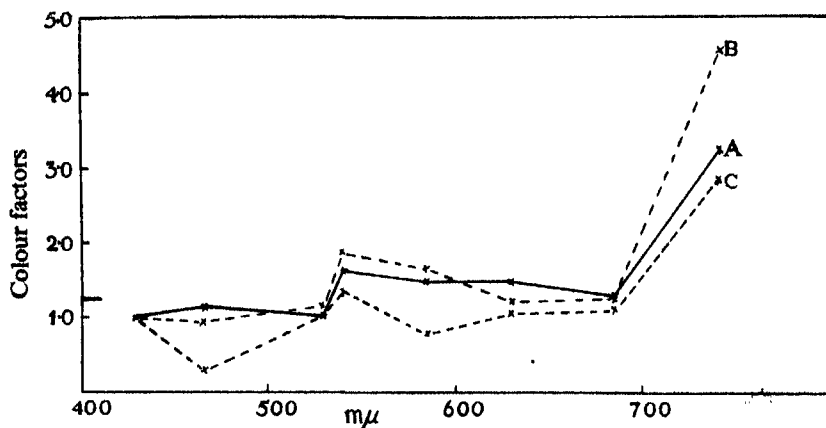


FIG. 4—Colour factors relative to grey sky light at Bristworthy Wood, A, $\delta = 2.7$ and B, $\delta = 2.0$, 1 August, 1935; also at holm oak wood at Antony, C, $\delta = 2.6$, 2 August, 1935. Determined using selenium cell with colour filters.

THE STANDARDIZATION OF PHOTOELECTRIC CELLS FOR THE MEASUREMENT OF ENERGY

No really satisfactory method is known for the ready standardization of photoelectric cells for the measurement of radiation in absolute units. The chief difficulty is due to the relatively low temperature of all artificial sources, which renders their spectral energy distributions widely different from that of daylight. As the sensitivity of a cell varies greatly throughout the spectral range to which it is sensitive, its reading depends not only on the total energy in that range but also on its distribution, so that an energy-constant derived from a lamp determination is likely to be misleading if applied to the measurement of daylight. Our best chance is to break the spectrum up into blocks by the use of filters, so that errors due to variation of distribution within each block are reduced. We have described (1936, *b*) a method of obtaining constants in this way by means of a standard lamp at a certified colour temperature of 2360° K. The

energy in each spectral block is found from the figures published in the International Critical Tables for a black body, allowance being made for the fact that tungsten radiates relatively more energy in the visible spectrum than does a black body at the same colour temperature.

The method enables us to find approximate values of the energy-constants with comparatively simple apparatus, but owing to the unsatisfactory spectral distribution of the source and the importance of small errors in the certified colour temperature, it is doubtful whether these constants can be expected to have errors less than 10%. The constants derived from the lamp are given in Table V.

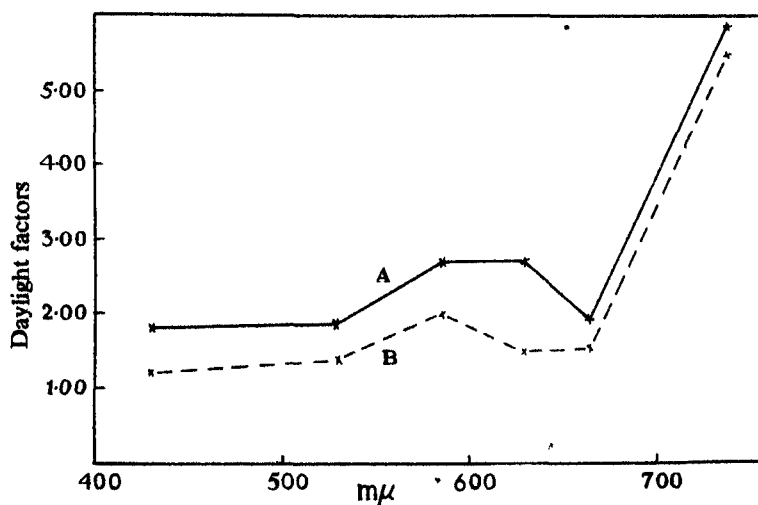


FIG. 5—Brisworthy Wood, 1 August, 1935; daylight factors plotted against wavelength. Curves A and B refer to the sites described in text.

In Table VI examples are given of the use of these constants in the study of the light in the open and in a wood. It may be seen that whereas the spectral blocks each contain about 1.5–2.5 milliwatts in the open, in heavy shade they are of the order of 20 to 70 microwatts.

The numerical values of Table VI have been used to ascertain the relative spectral composition of the grey light in the open and at the two sites in Brisworthy Wood. These have been plotted in fig. 6 and show that while the grey sky light is richest in blue, the light under the trees is richest in deep red and in green.

We desire to express our thanks to the Government Grant Committee of the Royal Society for assistance in the purchase of most of the instruments used. We are indebted to the Royal Dublin Society and to the Marine

Biological Association of the United Kingdom, Plymouth, for general laboratory facilities in connexion with the standardization of photoelectric cells.

TABLE V

Standardization of photoelectric cells, with colour filters, in terms of energy. The constants tabulated are in microwatts per square centimetre per microampere, and are for an equal energy spectrum which is nearer to daylight than that of a lamp. Thickness of filters 2 mm., but Corning green 4 mm.

Cell	Filter or filter difference	Range m μ	μ w. cm $^{-2}$ μ a $^{-1}$
Weston selenium No. 21104/2	BG 12	360-500	25.4
	GG 11-RG 1	500-600	4.30
	RG 1-RG 8	600-700	9.35
	RG 8	700-820	95
	No filter	360-820	9.6
	Corning green	500-600	46.5
	RG 1	600-760	13.4
	RG 7	760-860	1380
	No filter	400-760	7.5
Oxford Instrument Co., X 16, No. 498	RG 1	600-1100	148
	RG 8	700-1100	146
	RG 7	800-1100	261

TABLE VI

Photoelectric measurements of vertical radiation. About 4 p.m. G.M.T., 1 August, 1935; a calm day, grey sky, no sun. In open near Brisworthy Wood, Dartmoor, and at two sites in wood. The wood values are not simultaneous, so do not give δ which was separately determined.

Filters	Range	Vertical radiation in microwatts per sq. cm.		
		In open	In wood, $\delta = 2.7$	In wood, $\delta = 2.0$
BG 12	360-500	2528	39.6	19.0
Corning green	500-600	1782	28.4	15.3
GG 11-RG 1	500-600	2124	54.4	28.7
RG 1	600-760	2480	62.0	28.5
RG 1-RG 8	600-700	1572	35.9	14.7
RG 8	700-820	1505	72.6	51.9
RG 7	760-860	1285	24.4	51.4

SUMMARY

The optical conditions have been considered and convenient methods of measuring the photoelectric current in the field have been described. The wave-length sensitivity curves of the cells used have been plotted for a mean noon sunlight spectrum.

The illumination in shaded sites is best expressed, for ecological work, as the "daylight factor", namely the value of the vertical component of the illumination at any spot given as a percentage of the diffuse light in the open; this is nearly a constant.

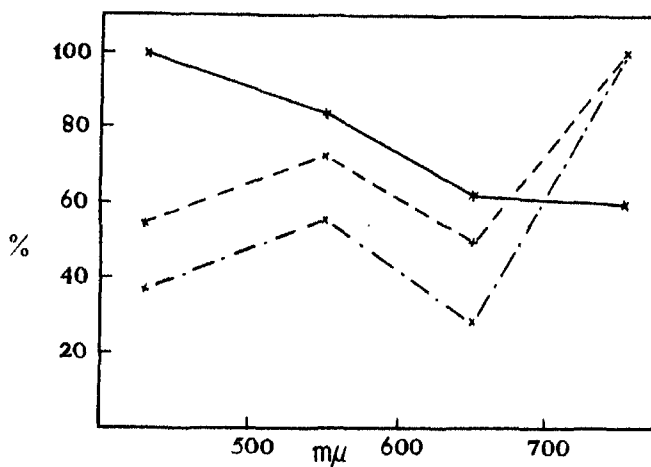


FIG. 6—The spectral composition of the grey light in the open at about 4 p.m. G.M.T., outside Brisworthy Wood, also of the light at two sites in the wood. The graphs are based on energy measurements made with the standardized photocells.

Burt vacuum sodium cells are serviceable for measuring daylight factors in even the deepest shade, but they only measure the violet and ultra-violet. It was found with a thin film caesium cell that different daylight factors were obtained in different parts of the spectrum. It has now been shown that such measurements can more conveniently be carried out using Weston selenium rectifier cells, calibrated for the curvature of the illumination-current relation, together with the glass colour filters of Schott of Jena. For the deep red and infra-red the type X cells of the Oxford Instrument Co. are useful. Photoelectric cells and colour filters must always be used under an opalized diffusing glass for work in daylight. A Moll solarimeter serves for the determination of the "radiation factor", namely the "daylight factor", taken over the whole spectrum. The ratio of the daylight factors for different spectral bands may be termed the "colour factor", by arbitrarily taking that for blue

light as unity. In a wood consisting mainly of *Fagus sylvatica* a number of sites examined showed daylight factors of 2–5%. In such heavy shade the colour factor is greatest for the deep red, and for green it is greater than for blue; there are indications of an absorption band in the near red. In such sites the radiation factor is about twice the daylight factor.

Selenium and type X 16 cells have been standardized to measure the radiation within broad spectral bands in microwatts per cm.² per micro-ampere. It was thus found that whereas grey skylight showed an intensity maximum at 430 m μ , light in heavy shade had its maximum at 760 m μ , with a minimum at 650 m μ , and a secondary maximum at 550 m μ .

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The Growth of *Chlorella Vulgaris* in Pure Culture

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A considerable number of investigations of the physiology of the alga *Chlorella* is in existence, but up to the present there seems to have been no detailed description of its growth cycle. The growth of a very similar alga, *Scenedesmus costulatus* var. *chlorelloides*, has been studied by Bristol Roach (1926, 1928) in some detail, so that the general course of the growth cycle could be inferred. Bristol Roach, however, was not able to include studies of dry weight gain and chemical composition in her analysis of the problem, and these properties clearly required consideration in any further investigations of algal growth. Moreover, as *Chlorella* has been used in this laboratory in studying the effect of various factors upon its metabolism, it was especially desirable that the effects of growth status and age upon its properties and chemical composition should be defined.

These considerations suggested the present investigation, which represents an attempt to study the growth of *Chlorella* in pure culture with particular reference to the relations between increases in number, in volume, in dry weight, and changes in chemical composition. It will be found that this aim largely determines the methods which must be used.

MATERIAL AND METHODS

The alga used was a strain of *Chlorella vulgaris* isolated by Hopkins and Wann and used by them in their investigations. We are indebted to Dr. E. F. Hopkins for this alga. It is maintained in pure culture on agar slopes, and grown in the standard medium described below before use. This medium is similar to that of Hopkins and Wann (1926). It consists of a mixture of equal parts of each of two solutions, A and B. Solution A contained to each litre of distilled water 0.4 gm. of anhydrous magnesium sulphate, 1.5 gm. of ammonium nitrate, 0.4 gm. of sodium citrate, three drops of ferrous sulphate (saturated solution), and 20 gm. of glucose. Solution B was a mixed potassium phosphate buffer solution, containing per litre 14.528 gm. of the dihydrogen salt and 4.646 gm. of the dipotassium salt. This maintained a p_H of 6.0, a value at which

maximum growth was observed by Hopkins and Wann. Twenty-five cubic centimetres of each solution were mixed in a 200 cc. conical Pyrex flask, with a cotton-wool plug, and sterilized by autoclaving for half an hour at 12 lb. pressure. When cool, each of a series of such flasks was inoculated with a known quantity of a sterile suspension of the alga in distilled water.

The cultures were placed around a series of 100-watt "daylight" lamps, each set being screened from the light of adjacent lamps. Illumination was continuous and the light intensity in each position was measured with a calibrated Bernheim photovoltaic cell (Pearsall and Ulliyott, 1933). The incident light intensity was 2500 ergs/sq. cm./sec. The cultures were kept in a small inside room of which the temperature could be maintained approximately constant with a daily fluctuation of about 1° C. Temperature was recorded on a thermograph, and for the present experiments it was $23 \pm 0.5^\circ$ C.

Each flask was shaken in a constant rotary manner in the morning and in the evening. This serves to prevent the formation of algal colonies and also, to some extent, to prevent the maintenance of local differences in the solution. The alga tended to settle into a layer on the base of the flask. This layer was about 2 cm. below the surface of the liquid. Undoubtedly gases could diffuse at an appreciable rate through the cotton-wool plug into the flask. A similar volume of molar potassium hydroxide solution in such a flask, for example, absorbed 10 mg. of carbon dioxide in two days under the same conditions. Nevertheless, it will be evident that some limitation of gaseous exchange to the alga may be possible. The removal of this limitation was not practicable for the large numbers of flasks required. The position of the alga in the flask also determines the method of measuring the light intensity, which was taken to be that falling on a horizontal surface, that of the photovoltaic cell placed below the base of a culture flask.

Counting—The numbers of cells present at any time were determined by withdrawing aseptically one or two cubic centimetres of the culture after thoroughly mixing it. The cell numbers were then counted by placing a drop on a Fuchs-Rosenthal haemocytometer. If necessary, their average diameter was determined using a micrometer eyepiece and oil immersion objective. The method of counting was to take the means of the numbers observed in three successive drops. It will be evident that two main sources of error may enter into such counts. The area of the haemocytometer examined may not represent the average number of cells in that drop of liquid, and the individual drops may not be repre-

sentative of the whole volume of liquid from which they are taken. Student (1907) has shown (for yeast cells) that the standard error of counting any one drop introduced by these factors equals the square root of the number counted. Hence if a reasonably large number of cells is counted in any one drop, the error will be proportionally smaller. Thus for more than 200 cells, say 225, the standard error may be of the order of 15, that is 6.66%. In normal practice then, we counted more than 200 cells in each of three drops, the mean of the three counts being assumed to be the number of cells in the culture. The numbers obtained for twelve separate drops taken in the normal manner from a culture are

EXAMPLE I

Number counted	Means taken	% difference from mean of series	Means of pairs	% difference from series mean
218	—	—	—	—
209	216.3	-1.59	213.5	-2.87
222	—	—	—	—
230	—	—	226.0	+2.82
221	222.0	+1.00	—	—
215	—	—	218.0	-0.82
222	—	—	—	—
211	217.0	-1.27	216.5	-1.50
218	—	—	—	—
231	—	—	224.5	+2.14
217	224.0	+1.91	—	—
224	—	—	220.5	+0.32
219.8 mean				

given in Example I. If these are grouped in threes (in the order in which they were made) it will be found that the mean of any three differs from that of the whole series by less than 2%. If the counts are grouped in pairs the mean of the pairs is never more than 3% above or below the mean for the whole series. This latter procedure was used as standard by Bristol Roach (1926) and has been employed also by us in counting replicate cultures for chemical analysis. In the latter case the mean number taken was the mean of the counts on replicate culture taken, at least three in number. In such cases time was important, and hence the less accurate counting technique was preferable.

It seems possible, from this example, to conclude that the estimates of cell number are reasonably accurate. This conclusion is, however, dependent upon the number of cells present in the medium counted, for

it can only apply when over 200 cells are visible in the haemocytometer used, that is, in a volume of 3.2 cu. mm. The cell number is then of the order of 60 or more per cu. mm. With smaller numbers, of course, "Student's" rule must apply to each individual count, and it is necessary to make proportionate increases in the numbers of drops counted, so that the estimations become laborious.

These considerations apply only to counts in a single flask and, in the present case, it is necessary to consider also the variation between replicate flasks. Example II gives a general idea of this variation, and, in particular, serves to show that the degree of variation tends to be different at different stages in the life of the culture. These cultures are from a typical series comparable to that given in Table I. A and B were growing exponentially, B growing slowly (in the sense used on p. 459), while D and E had practically ceased growth.

EXAMPLE II—ESTIMATES OF CELL NUMBERS (PER CU. MM.) IN REPLICATE CULTURES AT DIFFERENT AGES, A TO E

Series	A	B	C	D	E
426	2600	11100	23000	22050	
522	2200	13650	25100	22400	
468	2680	12950	22600	24000	
508	2780	14400	23300	24550	
612	3430				
536	2990				
Mean	512	2730	13025	23500	23250
Standard deviation	21	356	612	955	525
S.D. as % of mean	4.1	13.0	4.71	4.07	2.26

These examples show very well that the variability between replicates is greatest when growth is rapid, notably in B, and falls to a low level in mature cultures (D and E, where E represents cultures 6 days older than D). In actual fact, we have not often observed variations as large as those in D, E being more typical for mature cultures. (*Cf. also example III, B.*) The causes of the higher variability in growing cultures can be recognized. In the first place, it is not possible in inoculating long series of flasks under aseptic conditions to maintain absolute uniformity in the inoculum number, even when mechanical methods of producing the inoculating drop are used. Our estimates of the variation in number give a *possible* variation of slightly less than 5% above or below the mean. (This does not apply to short series, where much greater accuracy is possible.) Secondly, Bristol Roach (1926) showed clearly that cell division in such cultures tends to be discontinuous, so that in replicate

flasks the number may be higher or lower depending on whether a burst of division has just taken place or not. Thirdly, our counts are not all done at precisely the same time when the cultures are being filtered and weighed. It is obviously necessary to count the number just before filtering and hence, depending on the rate of filtering, an hour or more may elapse before the last of the replicate flasks is counted. Finally, the rate of growth in these cultures is extremely high, the numbers increasing about three times in the course of a day. This must clearly accentuate any tendency to variability due to the above causes, when rapid growth is in progress, though the cultures tend to approach a much more uniform condition when growth is slow or has ceased.

It is clear from the above that for numbers below about 10,000 per cu. mm., a minimum of six cultures is advisable in order to obtain an estimate of the average cell number. In mature cultures a mean of three cultures will normally suffice.

CELL SIZE

When determinations of cell size were made, one hundred cells in each sample were measured with a micrometer eyepiece and oil immersion lens. *Chlorella* cells may, at times, be somewhat elliptical in shape and their sizes were therefore measured as mean diameters. The size variations may be very considerable, especially in slowly or non-growing cultures. During rapid growth, size variation tends to be smaller. This point is illustrated by the following figures.

Number of Cells in Each Size Class

										Mean and standard deviation
Size class diameter in μ ...	4	6	8	10	12	14	16	18	20	
Rapidly growing	4	27	56	11	2	—	—	—	—	7.60 ± 0.15
Mature	5	16	19	16	13	11	10	8	3	11.02 ± 0.43

The important point in the determination of size is, what is the degree of accuracy of the means by the method employed? Actually, when the method was under consideration, it was found that the mean sizes could be estimated to within 1% by this method. Thus, in five successive drops from the same culture, average diameters of 7.60, 7.48, 7.60, 7.50, and 7.56 were obtained. Mean 7.55, extreme deviation 0.07. Further experience in practice, however, has shown that variations greater than this are observed if the measurements are made on different days or by different observers on the same day. The "error of the day" may clearly

approach 3% of the mean. It is well illustrated by Example III, of the extreme estimates made on different days on the same preserved culture.

EXAMPLE III

Observer	Mean diameter	
	Lowest estimate	Highest estimate
A	6.62	6.96
B	6.64	6.98

Thus both observers obtained similar maximum and minimum figures on some of the days—but their *range* of estimates might vary considerably. It is found in practice that this “error of the day” is not removed by using more size classes or by measuring more cells. It is obviously due to the observer’s estimate of size, under high magnifications. As it affects the estimates on small cells most, the examples given have dealt only with small cells. With larger cells the variation is of the same order of size ($\pm 0.18 \mu$), and it is therefore a proportionately smaller percentage of the mean size. In order to reduce this error of the day, the cell sizes for the later cultures in this work have been estimated from preserved material by one observer, as far as possible on the same day. The methods employed of fixing the cells produced no measurable alteration of size.

The importance of this fact lies mainly in its bearing on the estimates of the total volume of the cells in any culture. The total volume (V) of the cultures as given in the tables was estimated as

$$V = n \times 0.5236 \times d^3 \times 10^{-6} \times v,$$

where n is the cell number in 1 cu. mm., v the given volume of medium (in cu. cm.), and d the average diameter of the cells in μ .

In dealing with cultures of *Scenedesmus chlorelloides*, Bristol Roach (1926) used the total volume as a criterion of growth in preference to cell number. This was because, for example, if the cells had just divided, one might have $2x$ small cells instead of x large cells, although the total volume of protoplasm might not be greatly different. Hence for cultures growing discontinuously, total volume may give a more uniform growth curve than does cell number. Her experience and our own for cultures growing discontinuously may serve to justify the use of total volume in such cases. On the other hand, it is necessary to point out that the volume has to be calculated from the product of the estimated cell number and the *cube* of the mean cell diameter. Any errors in the diameter

estimate are thus multiplied and the magnitude of the error in measuring cell diameter is thus very important. If the possible error in the size determination is 1%, the possible error in the volume estimate from this cause will be less than 3%.

It may be mentioned also that the variation in volume of replicate cultures always tends to be less than the number variations during rapid growth. It will be shown that, at this time, cell size tends to decrease as number increases. Hence, of a series of replicates, one with a sub-normal number tends to consist of larger cells and to possess a volume nearer the average. The opposite condition applies to numbers above the average. A series of results given in Table II, day 5, serves to illustrate this point.

We made use of the technique described, which is essentially that of Bristol Roach (1926), as we intended originally to use the volume estimates incidentally for comparison with her work. In view of conclusions which will be indicated later (especially in regard to Table VIII), a more rigorous analysis of the volume estimates has become desirable. A simple calculation will show that when the range of diameter variation increases, the real cell volume will tend to rise as compared with the volume estimated from the mean diameter. Thus three cells each with a diameter of 6 μ will have a real volume the same as their estimated volume, but if their individual diameters were 5, 6, and 7 μ , the real volume would be to the estimated volume as 684:648. Thus the estimated volumes of cells varying in diameter tend to be underestimates and, in particular, it will follow that volume estimates for mature populations, which have a greater range of diameter variation than rapidly growing cells (p. 455), are likely to be underestimated more considerably. The conclusions drawn from the volume estimates are limited on account of this fact. A revision of a considerable body of data shows, however, that more detailed estimates would not materially alter the conclusions as to growth rates derived from volume estimates. In regard to Table VIII any increase in diameter variation in the later stages of growth (such as is possible) would tend to strengthen the argument advanced, although apparently in Series I no material change in cell diameter was taking place over the critical period from day 7 to day 17.

The variation between estimates of cell number, volume, and dry weight made under standard conditions is given in Example IV. These estimates are averages for five cultures of growing cells, A and B, and for three cultures in mature cells, C (excluding volume).

It seems safe to assume from these figures that the estimates are likely to agree to within 3%. On this basis, it has been the practice to use for

analytical work means derived from a minimum of six cultures with rapidly growing cells and three for mature cultures. At least four cultures were used for slowly growing cells. Larger numbers of replicates were frequently harvested at one time when this was necessary to get the weight of material required for analysis. Apart from the data for chemical composition, however, most of the conclusions drawn can be checked by working with single cultures, examples of which are therefore included in this paper.

EXAMPLE IV—COMPARISON OF AVERAGE ESTIMATES FOR REPLICATE CULTURES

	Cell number/ cu. mm.	Per culture	
		Volume cu. mm.	Dry weight mg.
A	2770	24.0	9.50
	2860	24.1	9.72
	2850	24.3	9.65
B	4900	27.2	13.20
	4960	27.5	13.02
	4870	27.3	12.90
C	23300	—	188.4
	23800	—	189.3
	22600	—	186.8

I—CHANGES IN NUMBER, VOLUME, AND DRY WEIGHT

The amount and velocity of growth shown by *Chlorella* under the conditions employed here may be illustrated by the following series of results. These series are representative of many others, each of them has been repeated, and they are given to illustrate the range of methods employed and the degree of variation in the results. The latter is important as the cultural operations here described have extended over four years. Series I is given as in many respects the most complete in our possession. Series II represents a type of observation much more frequently made. Each of these cultures was inoculated with actively growing cells from a similar culture growing under similar conditions.

The results of Series I are given in Table I and graphically in fig. 1. The data measured are the cell number per cu. mm. of solution, the average cell diameter, and the average dry weight of the cells in each culture. The volumes and dry weights (averages for a number of flasks) are similarly expressed as the amounts present in 10 cc. of solution. The

actual weights measured were at least twelve times as much as those given (see Table X).

The results are expressed graphically as the logarithms of the numbers, volumes, or dry weights.

This method of expression brings out very well the fact that growth is exponential in character, the logarithm of the number, volume, or weight increasing in a linear manner with time. Fig. 1 shows that the develop-

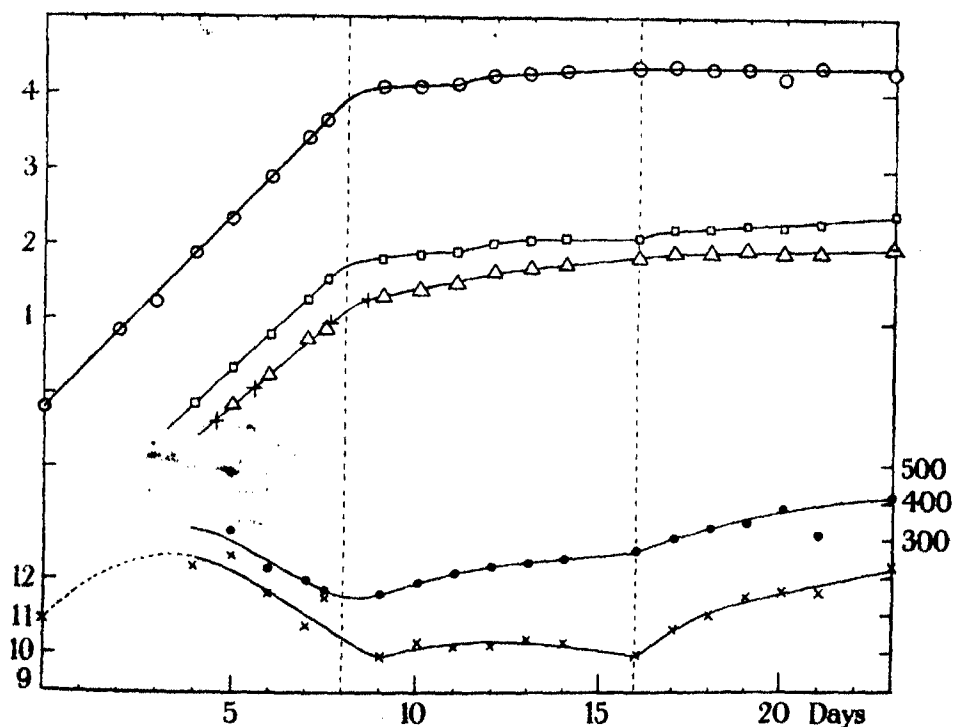


FIG. 1.—Logarithms of amounts of growth in Series I in relation to time. ○ Cell number per cu. mm. of medium; □ volume (cu. mm.) of cells in 10cc. of medium; Δ dry weight of cells as mg. per 10 cc. of medium; ● relative dry weight as mg. per 10⁶ cells; × mean cell diameter in μ .

ment of these cultures can be divided into (a) a period of rapid (exponential) growth to about 6-7000 cells/cu. mm., (b) a succeeding period of slow growth, (c) a period of cell extension. The last period starts about day 17, and it is marked by a considerable increase in the diameter of the cells and the volume of the cultures, although the dry weight and numbers remain fairly constant. It may be noted also that there is a considerable fluctuation in cell number during this period, particularly noticeable on days 19, 20, and 21. At this time the *extreme* variation of

the numbers in different cultures on any one day for this series is about 1300 above or below the mean (*see* Example II, E, and Example IV). A difference in number of 3000 should therefore be quite significant, and the results for day 20 must be regarded as significantly lower than those for days 19 or 21. This fluctuation in number is a constant feature of mature cultures, and usually there is a tendency towards an increase every

TABLE I—SERIES I

Day	Cell number/ cu. mm.	Average diameter in μ	Per 10 cc. of solution		Relative concentration mg. dry weight/cc.	Relative dry weight mg./10 ⁹ cells
			Volume in cu. mm.	Dry weight mg.		
0	0.6	10.90	0.0041	—	—	—
2	6.9	—	—	—	—	—
3	16.2	—	—	—	—	—
4	71.5	12.34	0.703	—	—	—
5	202	12.60	2.11	0.673	319	333
6	729	11.56	5.91	1.66	281	228
7	2615	10.74	17.0	5.10	301	195
7.5	4045	11.50	32.1	6.94	217	172
9	11850	9.86	60.9	18.6	305	157
10	12170	10.30	69.8	23.0	329	189
11	12790	10.18	71.7	27.3	386	214
12	17320	10.18	95.7	39.9	416	231
13	18490	10.38	108.1	43.5	403	236
14	20360	10.26	115	50.4	439	247
16	22150	9.88	112	60.9	543	275
17	23400	10.70	149.6	71.5	479	306
18	20780	10.96	143.4	70.0	487	336
19	21960	11.46	173	77.7	450	346
20	18600	11.66	155	71.6	462	384
21	22270	11.48	176.5	70.5	399	316
23	19840	12.32	193	82.3	427	416

second day. There is evidence that some of the cells die, and that about every two days a small burst of division takes place. In much older cultures than these, the number of cells remains more nearly constant, the differences observed not being significant.

Considerable fluctuations of dry weight may also occur from day 16 onwards. If our estimation of the variation of these older cultures is correct (p. 458), these daily differences should be quite significant. This

assumption is borne out in a striking manner by the similarity between the results for Series I and a check culture (Table X). For equivalent days, the following figures for dry weight (mg.) were obtained:—

Series I, days 16 to 19	265·2	296·2	287·0	314·5
Check series	263·8	298·3	285·3	317·3

The general agreement is remarkable, considering that these results were separated by an interval of five weeks.

The method of presenting the results graphically as the logarithms of cell numbers or volumes is necessary in order to bring out the exponential character of the growth process. At the same time, this method serves to reduce the importance of the second phase of slow growth, although this actually results in an increase of cell number to about four times that present at the end of the exponential phase. This method is justifiable because the *rate of growth* in the later slow phase of growth is very small in comparison with that during the exponential phase, and the later phase is therefore physiologically less important.

So far as the exponential phase of growth is concerned, we may say that it shows that growth proceeds according to the equation

$$\log N_t - \log N_0 = kt,$$

where N_t is the number at time t , N_0 the number initially, and k is the logarithmic growth rate. This value, k , may be calculated or it may be estimated as the slope of the line obtained when the logarithm of the growth unit measured is plotted against time. We may estimate k for increase in cell number as being 0·50–0·51. That is to say the cell number increases about ten times in two days, or rather more than three times in a single day. The rate of increase of volume is distinctly less than this as the value of k is of the order of 0·47. There is a difficulty in this latter estimation. The initial value on day 0 does not lie on the line joining the points from days 4 to 7·5. In such cases, failing precise evidence as to the intermediate stages, it seems to be better to take a value based on the slope of the experimental points (days 4 to 7·5) since this must represent a steady state of growth. Judgment as to what happens in the earlier stages of growth must necessarily be reserved.

As dry weight determinations can only be carried out on a few days at the end of the exponential phase, we give also in the figure the results for another similar series (Table X) done immediately before Series I and under the same conditions. The average rate of increase in the logarithm of the cell number of this earlier series was 0·50, that is,

essentially the same as Series 1. The logarithmic rate of increase of dry weight in these two series is of the order of 0.41 to 0.43. The low dry weight on day 7.5 reduces the value of k to 0.41, the remaining results indicate the higher value. We may assume 0.42 ± 0.01 as a first approximation.

The values of k obtained from these results are hence distinctly different from one another. They are 0.51 for number increase, 0.47 for volume increase, and 0.42 for dry weight increase. It will be noted that the growth rate is much higher on a number basis than on the volume or dry weight basis. This means that the volume per cell and the dry weight per cell must decrease steadily throughout the exponential phase of growth, or at least throughout the later part of this phase, to which the data refer. In necessary agreement with this, an examination of the figures for cell diameter shows that a general decrease in cell size occurs during exponential growth.

In the last column of Table I are given data for the dry weight of a thousand million cells (relative dry weight) which show that this quantity also decreases. It reaches a minimum along with cell size, at or just after the end of the exponential phase of growth. Furthermore, if the preceding column in Table II is examined, it will be seen that the dry weight per unit volume of cell material (relative concentration) also decreases to a minimum at the end of the exponential phase of growth. This means that the concentration of solids in the protoplasm is least at this period. There is, therefore, a steady decrease in cell size, in cell dry weight, and in relative concentration during at least the later part of the phase of exponential growth. It would appear to be possible that all of these may be related to the decreasing concentration of the external solution, a point which will be considered later.

The relative dry weight (dry weight per thousand million cells) rises during the period of slow growth to a figure higher than any observed during exponential growth. During the final period of extension (day 16 onwards), there is a further marked rise in relative dry weight, though not in the relative concentration of dry matter per unit volume. The rises in relative dry weight when rapid growth has ceased must presumably be related to the maturation processes going on in the cells and to storage of reserve substances.

The evidence just considered as to the difference in the value of k when based separately on cell number, volume, or dry weight raises the problem as to which of these is the best criterion of growth. Clearly k based on volume is most nearly the mean of the three estimates. Further, the variations in the values of k on these bases are clearly the result of the

general tendency towards a reduction of cell size during exponential growth. If it were possible to get cells maintaining a constant size during exponential growth, then it might be expected that the rates of growth of cell number, of volume, and of dry weight would be essentially the same. The data in the following tables (Series II and III) represent the cultures in which this condition was most *nearly* reached in our observations—although in no cultures have we observed the cell size to remain constant. It has always shown a decrease during rapid growth.

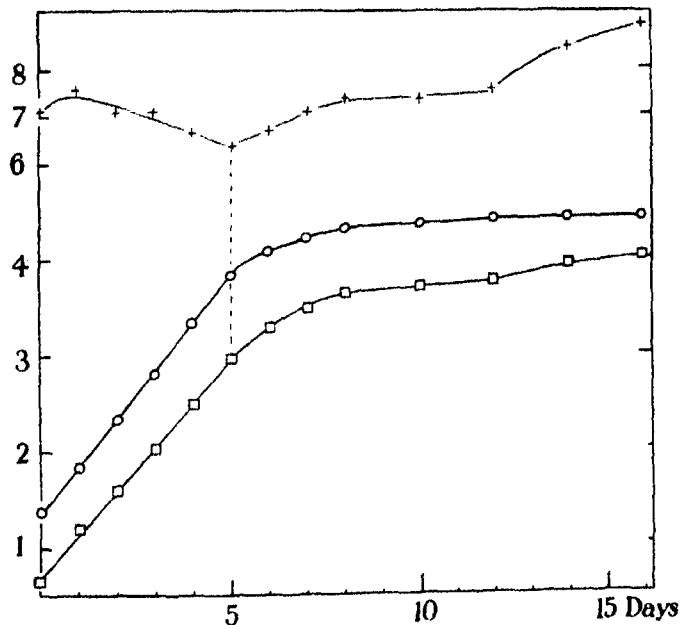


FIG. 2—Logarithms of cell number (per cu. mm.), ○, and of volume of cells, □, as cu. mm. per litre of medium; +, mean cell diameter in μ .

The cultures given in Series II and III were carried out more than two years after Series I and they are fairly typical check cultures, of which we have a number, a necessary feature of long-continued cultural operations. It will be noted that the cells used in these cultures are of much smaller average size than those used in Series I. While it is impossible to discuss this matter in detail here—it may be pointed out that if the average size of cells decreases during exponential growth, the result of growing the cells rapidly in a long succession of cultures may be that the average cell size will decrease, if the succeeding inocula are all taken during the later exponential phase. It is hoped to discuss these changes more fully in a later communication.

Series II gives data mainly for cell numbers and volumes, although there are dry weight data which allow the calculation of the average rate of dry weight increase. The data here are for counts on a single culture, checked by observations on companion cultures. As the daily removal of solution for counting reduces the volume of culture solution rapidly, the later counts in this instance (days 8–16) have been made on a previously untouched culture, as have the dry weight determinations. For further study of the dry weight changes of cells of this type, Series III must be consulted.

The main features of Series II exactly reproduce those of Series I (see fig. 2). Exponential growth continues until cell number reaches between 6–7000 cells/cu. mm. The number subsequently increases slowly until it is about four times this number. There is a final period (days 14–16) of marked size increase. The variation in cell size is proportionately less in this series than in Series I, and cell diameter shows, moreover, a steady increase during the period of slow growth which is not observable in Series I. There is some variation in this feature in our records, but most cultures show a general and small increase in average cell diameter immediately after exponential growth has ceased.

The rates of growth in Series II resemble those of Series I, and there is reasonable agreement between the values for the various cultures. Thus on day 5 the following *average* values for k were observed in different cultures.

Culture	Cell number	Volume	Dry weight
Main series	0.491	0.463	—
A	0.492	0.464	0.435
B	0.499	0.466	0.437
C	0.484	0.461	0.434
D	0.492	0.463	0.435

The changes in the relative dry weight of the cells cannot, of course, be followed in this type of culture, although the low value of k for dry weight increase is evidence that the dry weight did not on the average increase as rapidly as did cell number and volume. To illustrate the actual course of the dry weight relations in this type of culture, recourse must be had to Series III, in which the data are collected from various cultures of this type in which material was being prepared for chemical analysis. In some of these small-celled cultures we followed numbers, volumes, and dry weights by harvesting a single flask in the series on each day. These were done at irregular times in many cases, and, of course, the possibility of variation between cultures is against the use of such

isolated observations as continuous series. They can, however, be used to compare the changes of dry weight in relation to cell number or volume. In such series, a large number of cultures (fifteen or twenty) was harvested on the day for which chemical data were required. Some of these large harvests are included, as when the condition of the original inoculum is known they can be used to calculate average growth rates, with con-

TABLE II—SERIES II

Day	Cell number/ cu. mm.	Cell diameter in μ	Volume/ cu. mm. in 10 cc. of solution	Check cultures— number/cu. mm.	
				A	E
0	24	7.12	0.0452	24	—
1	69	7.60	0.159	70	—
2	216	7.09	0.404	204	—
3	555	7.10	1.04	634	—
4	2080	6.62	3.16	2180	—
5	6840	6.38	9.31	6920	—
6	12600	6.70	19.80	—	13400
7	16650	7.12	31.5	—	16900
8	21300	7.39	45.1	—	19800
10	24200	7.41	52.6	—	23600
12	26100	7.59	59.7	—	26200
14	27050	8.44	84.9	—	27250
16	27700	8.90	104	—	28100
5A	6920	6.38	9.42	—	—
5B	7500	6.26	9.67	—	—
5C	6290	6.51	9.10	—	—
5D	6880	6.38	9.36	—	—
10D	23600	7.48	51.7	—	—
16E	28100	8.78	100	—	—

Dry weights, mg./10 cc. of solution:—

Day 0, 0.0265; Day 5, A 3.83
B 3.97
C 3.81
D 3.87

siderable accuracy. It is known for all the cultures in this series that the inoculum was of the order of 3 to 5 cells of about 7.0 μ in average diameter. Other details are not available except in the cases given.

The main feature shown by these results is the change in the relative dry weight at different stages in the growth cycle. Growth may be regarded as exponential up to about 6000 cells per cu. mm., and all cultures below this are growing rapidly. The results can be expressed

graphically on a rough time scale if the relative dry weight is compared with log. cell number. It is plain, in any case, that the relative dry weight tends, as in Series I, to fall to a minimum value (near 50) at the end of the exponential phase (about 6000 cells/cu. mm.). For cell numbers between

TABLE III—SERIES III

Day	Cell number/ cu. mm.	Cell diameter in μ	Per 10 cc. of medium		Relative concentration mg./cc.	Relative dry weight mg./10 ⁶ cells
			Volume cu. mm.	Dry weight mg.		
0	3	6.90	0.0051	0.002	392	67
5*	615	6.86	1.08	0.485	449	79
7†	6450	6.33	8.60	3.23	375	50
0	4.7	7.21	0.0092	0.0034	370	72
6†	2795	7.07	5.15	2.06	400	74
†	5800	6.21	7.27	3.04	418	52
(10)‡	13750	7.24	27.4	14.3	522	104
(15)‡	28400	7.73	68.6	36.1	527	127
(25)‡	32800	8.61	125.4	56.0	447	177
	165	7.49	0.362	0.302	834	183
	219	—	—	0.298	—	136
	242	8.08	0.669	0.315	472	130
	825	7.40	1.745	0.727	417	88
	965	—	—	0.831	—	86
	1200	6.80	1.975	1.17	592	97
	2105	—	—	1.52	—	72
	6440	6.33	8.55	3.40	397	53
	8400	6.74	13.5	5.65	435	67
	13350	7.06	24.65	13.4	543	100
	19450	6.64	29.8	14.1	472	72
	21700	6.78	35.3	17.6	498	81
	22750	7.50	50.2	24.6	490	108
	26400	7.72	63.7	29.1	456	110
	28950	7.69	69.0	34.3	497	118

* 20 cultures.

† 15 cultures.

‡ 5 cultures—times comparable to Series I but not to other cultures in this table.

600 and 3000 per cu. mm., the observations (two of them for large numbers of cultures) show values above 0.072 mg. per million cells. The three single cultures containing about 200 cells/cu. mm. all show high relative dry weights, but in view of the possibility of error in such small weights (about 1.5 mg.) too much importance should not be attached to the exact

values of these figures. After exponential growth has ceased (above 7000 cells/cu. mm.), there is evidence of a general, if irregular, increase in relative dry weight which, by the time growth has ceased, is more than twice the value found at 6000 cells/cu. mm.

Data are also given in this table for the relative concentration, that is, dry weight per cu. mm. of cell volume. Little consistent variation in relative concentration was observed in these cultures, but it may be noted that this quantity tends to reach a minimum value of 400 or less towards the end of the exponential period of growth. This minimum of concentration was much more clearly shown in Series I. We may perhaps conclude that while there is a tendency for minimum concentration of dry matter to occur at the end of the logarithmic phase of growth, this tendency is by no means always so clearly shown as in Series I. On the other hand, reduction in cell size, and a decrease in the relative dry weight, appear to be characteristic features of the later stages of rapid growth.

The average logarithmic growth rates may be calculated for the first five results in Series III. The values of k for number, volume, and dry weight are:—

Days	Number	Volume	Dry weight
0-5	0.462	0.465	0.477
0-7	0.476	0.461	0.458
5-7	0.501	0.450	0.411
0-6	0.462	0.458	0.464
Mean	0.475	0.459	0.452

Because of the small variation in size of these cells, the results show less difference between the different values of k for any one time period. The results for days 0-6 are particularly interesting as the cells on day 6 were practically of the original average size. Thus k is similar for number and volume increase and also for dry weight increase. Clearly this must be an accidental result in the sense that it depends on the accident of harvesting the cells on the day when they were of the initial average size.

The comparison of the average values of k for the three time intervals of the same cultures, days 0-5, 0-7, and 5-7, shows that while the rate of volume increase remains nearly constant, the rate of increase of cell number rises, particularly after day 5. In contrast, the rate of dry weight increase falls markedly after day 5. These results are of interest as they suggest that the exponential rate of growth of cell number or of dry weight does not remain absolutely constant during the period of rapid growth. There are other indications that this is the case. In each of

the three series of results given there are signs that the average cell diameter increases during the first day or two after inoculation. Series I shows a definite size increase at this time, but most of our records resemble Series II and III more closely in giving smaller indications of size increase, usually between 0.5 and 1.0μ . The difficulty is that the smaller differences, such as are shown in Series II and III, may not always be significant, as in addition to the measuring error, which may be $\pm 0.1 \mu$ (see p. 455), we have to take into account the rather rapid fluctuations in size which are possible in rapidly growing cultures. Our experience suggests that these approach at least 0.2μ on either side of the mean size. Thus a size difference of 0.5μ would be only just significant. On the other hand, we have constantly observed an increase of size during growth immediately after inoculation, and this is undoubtedly significant when large cells are employed. We incline, therefore, to the belief that there is an increase in cell size after inoculation, and in a similar way the evidence suggests that there is also an increase in relative dry weight. In view of the fact that both size and relative dry weight subsequently decrease, it follows from these assumptions that at most only one of the three criteria of growth—number, volume, and dry weight—can give a steady exponential rate of growth. If, as in these results, the rate of volume increase remains approximately constant, then the rate of number increase must rise as the cells become smaller and the rate of dry weight increase must fall as the relative dry weight of the cells declines towards the end of the period of rapid growth.

It may perhaps be of interest to point out that *after* the period of rapid growth, the rate of increase of dry weight is greater than that of cell number, because at this time the relative dry weight rises steadily.

We believe that these changes in dry weight are highly significant features of the growth of these cultures. It is usual to adopt the view that the cessation of rapid growth in cultures of this type is due mainly to exhaustion of the nutrient medium. If this is the case then a decrease in cell size and in relative dry weight would appear to be the logical results of the depleted nutrient supplies. The decrease in cell size would result in a higher ratio of surface to volume, and hence would permit rapid absorption of dissolved substances to continue for a longer time. This point of view will receive further attention at a later stage. For the present, it seems desirable to distinguish tentatively at least two parts of the exponential growth period. Firstly, there is an earlier part during which cell size and relative dry weight are increased or at least maintained at the original level, and, secondly, the later phase of exponential growth is marked by declining size and relative dry weight. When rapid growth

has ceased, both relative dry weight and usually cell size increase again. Hence we may assume that, in slowly growing cells, absorption of material has again overtaken utilization—so that accumulation of dry weight is possible. Still further accumulation is evident in the last stage—of cell extension—when the relative dry weight rises again.

II—MORPHOLOGICAL CHANGES IN THE CELLS DURING GROWTH

Consider next the mechanism of growth in this alga and such evidence as there is about the increase in number. *Chlorella* generally divides into two, four, or eight daughter cells, which remain for a time surrounded by the cell wall of the parent cell. It was observed at an early stage in the work on this alga that a good deal of variation might be observed, in different cultures, in the numbers and types of dividing cells present. Accordingly the proportions of these types of cells have been determined in certain cultures, and typical results are given in Table IV.

It is simplest to obtain first a general idea of the variability in cell division. In order to convey this idea, we have constructed Table IV, showing the number of dividing cells (as a percentage of those present) in four typical cultures grown at different times, in relation to the cell number. We do this because it is easy to draw erroneous conclusions from a single series. Bearing in mind that exponential growth ceases at about 6000 cells/cu. mm., it is possible to compare the proportions of the different forms of dividing cells seen at various stages in exponential growth and in the subsequent period of slow growth.

This table at once shows the great variability exhibited firstly in the total percentage of dividing cells, and secondly in the proportions of cells containing two daughter cells to those containing four or eight. It seems, in fact, that only two general conclusions are possible. In the first place, the first ten records show that when the cell numbers are low there is a definite tendency for a low proportion of four-celled divisions. Secondly, cells containing eight daughter cells usually only appear after exponential growth has ceased. Both of these conclusions appear to involve the same fundamental change, which is that the daughter cells escape from the parent cell more easily in young cultures or more slowly in old cultures. This development is evidently progressive and, in passing, it may be noted that it gives visual evidence of our assumption that two phases can be distinguished in the exponential period of growth.

Apart from this developmental change, the variability in the proportions of visible division stages appears also to be a fact of great interest. It contrasts very markedly, for example, with the case of *Scenedesmus*

TABLE IV
As percentage of cells examined

Cell number/ cu. mm.	Number of daughter cells			Total dividing
	2	4	8	
39	7.0	0.5	—	7.5
44	3.6	0.5	—	4.1
64	6.0	1.2	—	7.2
66	2.4	1.4	—	3.8
80	3.5	0.7	—	4.2
100	3.7	0.5	—	4.2
100	4.6	0.8	—	5.4
128	3.2	0.5	—	3.7
165	3.7	0.5	—	4.2
219	4.0	0.8	—	4.8
242	3.6	2.6	—	6.2
268	4.7	1.0	—	5.9
270	6.2	3.1	—	9.3
615	7.4	2.0	—	9.4
825	4.3	0.6	—	4.9
960	6.6	2.0	—	8.6
965	4.6	1.3	—	5.9
1100	3.2	0.5	—	3.7
1200	5.6	1.7	—	7.3
2105	3.8	0.7	—	4.5
2795	5.1	0.9	—	6.0
3170	4.0	3.0	—	7.0
4020	3.7	1.2	—	4.9
5800	2.8	0.9	—	3.7
6450	4.7	0.4	—	5.1
8140	3.0	1.3	0.1	4.4
8400	1.9	1.6	0.3	3.8
10400	1.4	1.0	—	2.4
10650	3.5	0.4	0.2	4.1
13350	5.7	4.0	1.6	11.3
18600	3.5	3.9	1.7	9.1
18640	2.0	3.5	0.2	5.7
19450	4.3	2.2	0.1	6.6
21700	3.0	1.1	0.4	4.5
22000	2.0	3.5	0.2	5.9

chlorelloides studied by Bristol Roach (1926) in which the ratio of cells containing two autospores to those containing four is usually three. In *Chlorella* under the present conditions no such constancy exists, and the observed irregularity can only be explained easily by assuming that the division stages appear somewhat intermittently in time and not continuously and uniformly. Thus, for example, during the later stages of exponential growth (1-6000 cells) the average percentage of division stages seen is about five. If dividing cells are produced in separate bursts, a higher percentage than five (e.g., seven) might represent the whole of one "crop" of dividing cells, the remains of a previous "crop" (presumably mostly four-celled), and the beginnings of a succeeding "crop" (presumably mostly two-celled). A low percentage of dividing cells, on the other hand, might represent the interval between two crops. Some such hypothesis as this helps to explain a number of facts observed in these cultures and also the variation in the proportion of cells containing two and four autospores. These variations would then also be greatest in slowly growing cultures, a fact actually observed and clearly shown in Table IV.

Attention may now be directed to the results in Tables V and VI which give the proportions of dividing cells of different types in Series I and II previously described. The numbers are based on counts of five hundred cells in each case. Series I gives averages for a number of replicate cultures, and Series II is for observations on a single culture. The wide possible limits of variation in any single culture should be borne in mind. Allowing for this possible variation, the general features of each series resemble those results already discussed, except in the presence in Series I (Table V) of some eight-celled division stages at the beginning of the growth cycle. This feature is occasionally observed when the inoculum included eight-celled stages, as sometimes happens when it is taken towards the end of the stage of rapid growth. In such a case, the eight- and often also four-celled units may persist with little change for a day or two, and it is not theoretically impossible that this mode of division may continue in a certain proportion of the cells.

The Mechanism of Growth

The data in Tables V and VI have been analysed in the following manner. We suppose that we can isolate, mentally at any rate, each group of dividing cells seen on examining a culture from the similar group which preceded it and from that which follows it. We can then think of each of these groups as representing a definite burst or unit of

cell division which must take a certain time to originate and to complete, and which will result in a definite increase in the number of cells. Thus if our group contains 50 dividing cells per thousand, of which 40 contain two autospores and 10 contain four autospores, then on completion of the division there will be 1070 cells instead of the original thousand. A number of such dividing groups may be expected to complete their division in the course of any time unit (e.g., a day) and the total number increase of the culture in this time will then be the sum of the additions made by the successive groups.

To economize words, we can call the products of each group of dividing cells a "division". The number of "divisions" per day (x) will then be given by $x(\log p) = \log N_1 - \log N_0$, where N_0 and N_1 are the numbers in the culture at the beginning and end of the day respectively, and p is the average number of cells produced by each "division", 1070/1000 in the example given above. Thus, taking as a further example days 6 and 7, Series I, p will be the mean of 1.141 and 1.199, i.e., 1.170, N_0 is 729, and N_7 is 2615, thus

$$x(\log 1.170) = \log 2615 - \log 729$$

$$0.068x = 0.554$$

$$x = 8.15.$$

That is to say, if $x = 8.15$, we assume that 8.15 divisions took place during day 6 to 7. The relative numbers of "divisions" for each day calculated on this basis are given in Tables V and VI.

But if we know the number of "divisions" each day, and also the number of cells seen to be dividing at any sampling time, we can also determine the proportion of the cells originally present which divides in the course of a day. Thus on day 6 to 7 the average number of dividing cells seen was 10.8%, and since there were 8.15 "divisions", the total number dividing in the course of the day must have been 10.8×8.15 or 88.1%. These percentages of the original cells dividing are given in the last column of Tables V and VI.

All the results available resemble those given in showing that on the average about 80-90% of the original cells divide in the course of a day. The average for Series I is 80.7 and for Series II it is 88.9%. In other words, we can assume that a large proportion of the population is active in growth, if each cell takes slightly more than a day to reach the dividing condition again.

If the population of cells at any time were composed partly of actively growing cells and partly of dormant cells we should undoubtedly find

that these groups would possess different average sizes (Richards, 1932). Hence the frequency curve giving numbers of cells in each size class would tend to be bimodal. This state has not been observed in cultures under the present conditions. We conclude, therefore, that all the cells are probably actively growing with a "generation time" of slightly more than one day. It may be pointed out also that only a small part of the time can be spent in a two- or four-celled condition. For, if there are fifteen "divisions" per day, each group of cells must show autospores for only about an hour and a half, possibly less.

These conclusions are necessarily of an approximate nature because no information is available as to the generation times of two- and four-celled autospores. If it takes longer to produce four daughter cells than to produce two (which seems reasonable), then the average generation time of the two-celled units will be less than that given above. In order to indicate the magnitude of the effects which may be produced by this factor, we may take a simple hypothetical example, assuming a population containing four two-celled colonies to each four-celled unit, which approaches the average condition during exponential growth. For the present purpose we shall also assume, as perhaps the most probable case, that each two-celled colony has a generation time of the order of one day, and that the four-celled units are produced by the further division of a two-celled unit after an additional day. Of the four two-celled units produced by one burst of division, then, one will persist for a day in this condition before dividing further. At any average time, then, we should see from a "division"—

- (a) 3 two-celled units separating soon afterwards,
- (b) 1 two-celled unit persisting for about a day,
- (c) 1 four-celled unit separating soon afterwards.

The assumed net production after the observation would then be (a) 6, (c) 4 cells, *i.e.*, $10 - 5 = 5$ net gain, instead of (a) (b) 8, (c) 4, *i.e.*, $12 - 5 = 7$ net gain as assumed above and in the tables.

The number of "divisions" per day as calculated will therefore be increased by the ratio $\frac{\log 7}{\log 5} = 1.21$.

The number of cells *really* dividing in this case is only $4/5$ of those seen with autospores. Hence the "percentage dividing" as calculated above will be altered by $4/5 \times 1.21 = 0.968$. It will be seen that the corrections made do not alter the order of magnitude of the result. We have therefore not attempted to correct our calculations for the possibly different

generation times of two- and four-celled units as we have been quite unable to obtain any evidence on the question of their comparative durations.

The two Series I and II to which attention is directed, differ in one interesting particular. Series I, composed of large cells, shows a progressive increase in the number of dividing cells visible as the stage of rapid growth progresses. This increase is not shown significantly in Series II, and the two series are typical of the extremes seen in our cultures.

While an increase in the apparent number of dividing cells has only been observed in large-celled cultures, we think it premature to regard this feature as characteristic of such cultures, particularly in view of the manner in which the proportions of dividing cells visible may fluctuate. The results of cultures like Series I are not, however, without interest, because if the visible number of dividing cells *increases*, it is only possible to get a uniform rate of exponential growth if the number of "divisions" per day *decreases*, as shown in Table V. This in its turn might imply either a progressive change to the slow growing state, or else that the cells were remaining longer in the autospore stage—so that more were visible at any moment. In the latter case, the next step would logically seem to be that eight-celled units would appear. These actually do appear when exponential growth is ceasing, and, as we have already shown, there is normally a transition during exponential growth from a condition when two-celled units predominate to one where four- and eight-celled units are common. Series I thus seems to represent a further expression of this tendency which may possibly be characteristic of large-celled cultures.

In this and Table V, the net gain in cell number per day is the number produced minus the number dividing.

Structural Changes Observed During Growth

While the figures tabulated in the preceding sections were being collected, many observations on the structural character and microscopical appearance of the cultures were made, which amplify the picture already drawn. Because any culture of *Chlorella* is normally heterogeneous, containing cells of varying growth status and size, our conclusions are not easily capable of numerical description except at an expenditure of time and of energy which would be disproportionate to the results obtained. But certain types of cells are found only at definite stages in the growth of the cultures and, starting from these, we can, with the figures already obtained, indicate the broad lines of structural progress. Of these

TABLE V—SERIES I

Day	Cell number/ cu. mm.	Per thousand cells					Number of "divisions"/ day	% dividing/ day
		Number containing daughter cells			Number dividing	Number produced		
		2	4	8				
3	16.2	59	15	0.1	74	179		
4	71.5	22.5	12	0.4	35	96	18.42	100.4
5	202	32	8	—	40	96	17.33	65.1
6	729	63	26	—	89	230	13.6	87.1
7	2615	91	36	—	127	326	8.15	88.1
7.5	4045	101	35	0.1	136	342	4.72	62.2
9	11850	70	27	3	100	272	4.15	49.0
10	12170	76	22	1.9	100	255	0.167	1.67
11	12790	38	19	—	57	152	2.37	18.4
12	17320	58	13	—	71	168	3.22	20.6
13	18490	51	15	0.8	57	168	0.622	3.98
14	20360	26	13	4	43	136	1.0	5.0
16	22150	48	8	—	56	128	0.544	2.69
17	23400	60	6	—	66	144	0.73	4.45
18	20780	59	8	—	67	150	—	—
19	21960	44	4	2	50	120	—	—
20	18600	30	6	1	37	92	—	—
21	20500	26	13	2	41	120	—	—
23	19800	12	4	2	18	72	—	—

features, the earlier ones are best observed in cultures for which a small inoculum (e.g., 1/cu. mm.) has been used. With large inocula (100/cu. mm.) the earlier stages are passed through more quickly, and, possibly, may even be absent.

Stage 1—On inoculation into new medium the cells apparently swell and rupture the old cell wall. The protoplasm is released and commonly appears to separate out into from four to sixteen smaller protoplasmic

units which may vary in size. The cells formed in this may have a very distinctive appearance. The chlorophyll is inconspicuous and the chloroplasts are small. Protoplasm is highly granular, and vacuoles are either absent or extremely small. A cell wall is indistinguishable and the whole cell may have a strikingly amoeboid appearance. Daughter cells appear to separate quickly as a rule, but occasionally a group of

TABLE VI—SERIES II

Day	Cell number/ cu. mm.	Per thousand cells					Number of "divisions"/ day	% dividing/ day
		Number containing daughter cells			Number dividing	Number produced		
		2	4	8				
1	69	38	5	—	43	96	17.7	85.9
2	216	41	13	—	54	134	11.4	69.0
3	555	54	13	—	67	160	18.7	117.8
4	2080	48	11	—	59	140	14.8	82.9
5	6840	37	16	—	53	138	7.57	39.3
6	12600	38	11	2	51	136	3.18	16.1
7	16650	33	14	3	50	146	1.57	12.35
8	21300	58	40	9	107	348	0.326	3.36
10	24200	62	31	6	99	296	0.197	2.05
12	26100	70	34	5	109	316	0.140	1.10
14	27050	39	7	2	48	122	0.156	1.20
16	27700	29	10	3	42	122		

cells may remain attached to one another. Cells in this stage are uncommon when the numbers exceed 100/cu. mm. and are possibly more readily formed from inocula from old cultures.

Stage 2—The early exponential phase of growth, as defined in the preceding tables, is characterized by the presence of cells with a very thin and delicate cell wall. The protoplasm is transparent, and much less granular, vacuoles are still normally absent or inconspicuous. A structure which appears to be the nucleus is very prominent. The chloroplasts cover about one-third of the cell and are rather pale and yellow-

green in colour. Division normally produces two daughter cells, and four-celled units are not common. This type of cell is common until the cell numbers approach 500 to 1000/cu. mm.

Stage 3—Above this number level, the predominant cell form normally changes to cells with a definitely thicker wall. From two to four vacuoles are present. The appearance of the protoplasm is not easy to make out, because the chlorophyll tends to become distributed over the whole surface. Its distribution is irregular, that is, not a continuous sheet. Cells containing four or eight daughter cells are more characteristic, the latter as the end of the exponential growth period is approached—about 6000 cells/cu. mm.

Stage 4—The appearance of the cells after exponential growth has ceased shows a further development of stage 3. The presence of numerous small vacuoles is very characteristic, as well as the thick wall. One really striking feature is the almost invariable presence of cells containing eight daughter cells and occasionally even more—probably sixteen. Furthermore, this period is characterized by the appearance of motile cells. The existence of these motile cells is not in doubt—nor is there any doubt about the time of their appearance, or the purity of the cultures in which they are observed. On the other hand, they occur sparingly in cultures containing very large numbers of normal cells. It is impossible to devise a method of finding them after any fixing or staining process. They are distinguished in a living culture by their activity, but at the same time they cannot be readily followed under an oil immersion lens. Their structural details must therefore remain obscure until a method has been devised of obtaining them in large numbers. They resemble in form and structure the cells of the early exponential phase, but they are small, 2 to 4 μ in diameter. From this size it is judged that at least thirty may come from a single cell. They possess two flagellae, probably, though not certainly, of equal length, one of which is normally distinct, the other difficult to see and perhaps partly coiled round the body.

Stage 5—After the cultures have reached their maximum of cell number they begin to show a general increase in size (see Tables I and II). The walls become thick and slightly lamellose, vacuoles are abundant, and the protoplasm spongy in appearance. Chlorophyll also appears to be abundant, and the cells look to be a darker green than at any earlier stage. This colour is gradually lost until after about fourteen days or more the cells tend to become yellowish—a characteristic feature of old or stale cultures. Cells showing division stages are present but less abundant.

It will be appreciated that these structural changes are gradual, and that the number limits suggested are by no means rigid. It is certainly possible, however, to recognize the growth status of a culture by its microscopic appearance.

The gradual increase in the thickness of the walls as cultures mature may possibly suggest a reason for the observed and parallel increase in the number of daughter cells produced. It is possible that the thicker walls in older cultures offer greater mechanical resistance to the separation of the daughter cells, so that four- and eight-celled units become more common.

Apart from the difference in the thickness of the cell walls at different stages, evidence for a physical difference in the walls is given also by the rates at which different cultures can be filtered. Cultures growing exponentially, and especially young ones, filter very slowly. Slowly growing cultures and especially mature ones filter relatively rapidly. The comparative differences in the time taken to filter any one culture are of the order of 20 to 5 minutes, depending on its age. We infer from these facts that the cell walls are softer and more gelatinous in young cultures, and that they become harder and less adherent in mature cultures.

The general sequence of developmental changes in these cultures is clearly as follows. The protoplasm becomes less granular, vacuoles appear, and first their size, and then their number increases. The cell wall, at first not distinguishable, increases in thickness and, probably, in hardness. The chloroplasts also increase in size and in depth of colour. The general resemblance between these changes and those which ensue in the growth and development of a green cell in higher plants is to us one of the impressive results of this investigation. As far as we know, it has not previously been emphasized.

III—CHEMICAL DATA

The chemical data given here are a selection from various analyses, and the selection has been made to indicate the facts which seem to be established, or to be worth further investigation. Before proceeding to consider the results, it may be desirable to indicate the basis on which the analyses were made. Because of the minute dry weights of the cultures in the earliest stages of growth, this phase of the growth cycle has not yet been examined, and the comparisons to be made here refer to the later exponential and subsequent phases. For cultures in these stages, we know that the ash content lies between 4·5 and 5·0% of the dry weight,

and little or no obvious correlation has been detected between this quantity and the growth phase. The fat (ether extractable) content of *Chlorella* is small (1 to 2% of the dry weight) and significant differences are not easily obtained. It therefore seemed inadvisable to include either ash or fats in routine analyses, especially as the estimation of these fractions would use up considerable quantities of material (500 mg. for fats). Further, although routine determinations of titratable acidity were made, these show no significant differences, and the results are not given in detail. The titratable acidity of *Chlorella* under the present conditions of growth is small, of the order of 0.2 cc. of N/100 acid per 100 mg. of dry weight. Finally, the determinations of nitrogen could be further simplified because nitrates are apparently not detectable in *Chlorella* extracts, while the amounts of ammonia are small, and have not been observed to show significant variations. From these considerations, it seemed that a reasonably complete picture of the chemical changes during growth might be obtained from estimations of total nitrogen, soluble nitrogen, and amino-nitrogen, together with soluble and insoluble carbohydrates. From the nitrogen estimations, we could then obtain by difference protein nitrogen and Rest nitrogen (soluble nitrogen other than amino-nitrogen). In regard to the carbohydrates, the estimations of soluble reducing substances before and after mild hydrolysis would serve to indicate the proportions of hexoses and disaccharides. The insoluble carbohydrates in *Chlorella* include starch, though from the iodine test it appears that this is often not very important. The wall in mature cells includes cellulose (micro-chemical tests), but it is difficult to be certain of the presence of this in young cells. A large part of the reserve carbohydrates in this alga can only be referred to the vague group included under the name of hemi-celluloses, and yielding on hydrolysis reducing substances which may be of a varied nature. We suspect that at least a part of this fraction is intimately associated with the cell wall (as in *Molinia*, Schellenberger, 1905) and that it may be responsible for the gelatinous character of this wall under certain conditions. After prolonged hydrolysis (as under), the walls of *mature* cells are still recognizable, and this residue is supposed to be cellulose. Hence we adopt the following scheme for carbohydrate analysis.

- (1) Total carbohydrates, after hydrolysis with 3% sulphuric acid at 100° C.

- (2) Starch, after hydrolysis of material with taka-diastase.

- (3) Soluble sugars by estimation on the extract.

From these estimates, total hydrolysable polysaccharides may be obtained as the difference between 1 and 3, starch as the difference between

2 and 3. "Hemi-cellulose" could be obtained as the difference between 1 and 2.

The methods used and also the limits of accuracy of the estimations are given in the Appendix. The methods had all been previously employed in estimations on *Chlorella*. From these earlier estimations, the general nature of the variations in chemical composition were apparent and the results given in the present paper represent series designed to confirm the earlier results and to coordinate them into a continuous picture. The estimates are actually made as percentages of the dry weight and they are given in this form in the Appendix. The results given are restricted to cultures already described in the first part of this paper. Series I is the most exhaustive we possess and is representative of large-celled cultures. As other results from check series add nothing to the information obtained from Series I, we give only in contrast a series of six typical results from small-celled cultures. These serve to show the extreme differences we have observed in the analytical data, and also to confirm the special features of the more rapidly growing cells, of which only one sample is included in Series I. Other data for these small-celled cultures are given in Table III, in which X and Y are respectively the cultures of day 6 and the subsequent entry, Z is the entry for day 7. The respective cell numbers are 2795, 5800, and 6400/cu. mm.

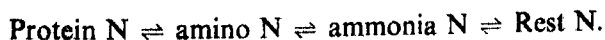
Chemical Changes—While the main object of the analyses was to determine the characteristics of the cells in the different growth stages, the consideration of this problem is best deferred until the general metabolic drift in the cells has been recognized. Series I is an attempt to get a continuous picture of these changes. It starts on day 7 with the cells growing exponentially, days 9–16 represent slow growth, while marked cell enlargement started on day 17. It has already been shown that the cultures are changing in dry weight per cell throughout their existence. Figures giving the chemical fractions as percentages of the dry weight or as weights per cell are therefore not necessarily a good basis for representing the changes in the constituents in the cells, and some other basis seems desirable.

We may suppose, for example, that as proteins form the structural basis of protoplasm, we may get a better picture of metabolic changes if we compute the results as weights (gm.) per unit weight of protein nitrogen (1 gm.). The results of such a computation are given in fig. 3 and Table VII to show the general drift during the maturation of the cultures. It will be seen that in general the proportions of insoluble carbohydrates increase steadily until about the 19th day. Sugars, on the other hand,

fluctuate in a marked degree. Hexoses are relatively high during rapid growth (day 7). They show a general fall until day 16 when they rise to their former level, *on this basis*. Disaccharides, in contrast, are low during exponential growth, but rise rapidly when it ceases until day 10–11—after which they fall steadily until day 19, when a second very marked period of accumulation develops. The final accumulation of disaccharide is apparently largely due to starch hydrolysis—since it is of the same order of magnitude as the observed reduction in starch at this time. These features have been observed in all cultures. The lines on the graph (fig. 3), for example, give the range observed in check cultures (large-celled); and the salient points are shown for small-celled cultures in Table VII.

The final feature of the curves to which attention is directed is the rise in the proportion of soluble nitrogen as age increases. This rise is particularly well marked when exponential growth ceases and also on day 17, when cell enlargement sets in. It may be evidence of a tendency towards protein hydrolysis which develops particularly at these two periods, or it may indicate a decrease in the rate of protein synthesis at these periods. The figures for amino N confirm the presence of this tendency while offering some uncertainty in exact interpretation. The suggestion of a final increase in amino N when disaccharides are also increasing (days 21 and 23) is borne out by other analyses we possess but cannot be regarded as conclusively proved.

On the other hand, it may be noted that the proportion of amino N to other soluble nitrogen is low initially but steadily increases as the cultures age. We may possibly assume that this means that amino-acids are used preferentially in protein synthesis (that is when the cultures are young), but, if we accept current theories of nitrogen metabolism, then an alternative explanation would be that the amino-acids are more easily converted to other forms of soluble nitrogen (Rest N) when the cultures are young.



The transformation of amino N to Rest N via ammonia is probably, partly at least an oxidation, since amino-acids accumulate in plant tissues when oxygen is absent (Mothes, 1926). Hence a tendency for accumulation of amino N in old cultures may mean a general slowing down of oxidation rates as the cultures age. This is quite in harmony with current ideas as to the changes of respiration intensity in developing plant tissues, and it will be shown subsequently that there is evidence for these cultures also that the respiration rate declines (p. 492).

We suggest, therefore, that in young cells amino-acids may be both condensed to protein and more rapidly oxidized. In old cultures, however, there is a greater tendency towards protein hydrolysis and a slower rate of conversion to other forms of soluble nitrogen.

It will be evident that it is not easy to distinguish, on the present basis of comparison, between the rapidly growing cells (day 7) and the enlarging

TABLE VII—CONSTITUENTS OF CELLS PER GM. OF INSOLUBLE NITROGEN

SERIES I

Day	Soluble N	Amino N	Hexose	Disaccharide	Starch	Total hydrolysable carbohydrate
7	0.098	0.025	1.08	0.51	4.35	14.65
9	0.117	0.033	0.84	0.78	5.1	17.7
10	0.120	0.040	0.84	1.01	5.3	17.9
11	0.120	0.043	0.74	0.96	5.2	19.1
12	0.128	0.047	0.87	0.86	6.4	23.9
13	0.124	0.039	0.78	0.70	6.7	25.9
14	0.122	0.051	0.73	0.74	7.25	25.0
16	0.120	0.048	0.74	0.59	7.05	26.95
17	0.153	0.063	0.97	0.62	7.8	31.35
18	0.141	0.050	0.98	0.69	8.8	28.0
19	0.139	0.049	0.86	0.59	10.4	25.2
20	0.145	0.051	1.005	0.89	10.1	29.6
21	0.152	0.062	1.05	1.56	9.8	31.1
23	0.145	0.063	1.04	1.73	9.2	30.6

SMALL CELLS—SERIES III

X	0.105	0.028	0.67	0.25	4.0	8.3
Y	0.102	0.023	0.54	0.40	3.75	8.1
Z	0.093	0.045	0.59	0.315	3.9	7.4
10	0.118	—	0.36	0.46	—	11.2
15	0.137	—	0.415	0.35	—	14.9
25	0.163	—	0.45	1.23	—	14.5

cultures (on days 17 and 18). While the former have a low soluble N, and low insoluble carbohydrate contents, the conditions in regard to soluble carbohydrates are closely similar at these periods on this basis of comparison (*i.e.*, as gm./gm. per protein N). Furthermore, a comparison (Table VII) between large-celled cultures and small-celled cultures shows that little similarity exists, on this basis of comparison, between the two

different types of culture, largely because the small-celled cultures are richer in nitrogen and poorer in carbohydrate. Their proportions of sugar per unit of protein N is therefore much smaller. One feature in which the two types of culture resemble each other is in the *proportions* of soluble N present. This is about one-tenth of the protein N when the cells are growing exponentially and it rises to about 15% when the cells are old. We may say that in the reaction protein N $\rightleftharpoons$ soluble N—the

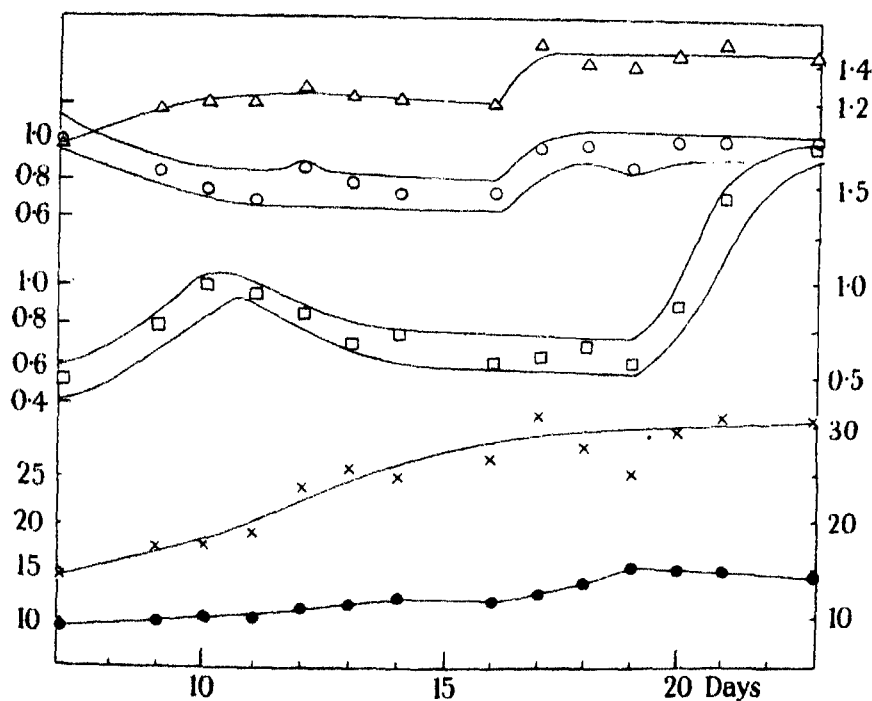


FIG. 3.—Chemical constituents present in Series I, per gm. of protein nitrogen. Curves for hexose and disaccharides show the range of variation observed in cultures of this type. Δ Soluble nitrogen; $\circ$ hexose; $\square$ disaccharides; $\times$ insoluble carbohydrate; $\bullet$ starch.

tendency is to go to the left in young cultures, to the right in old cultures. If we now make a similar comparison between the insoluble carbohydrates and the total sugars, then it appears in contrast that the tendency is for the insoluble carbohydrate to be low in rapidly growing cells, about nine times the sugars or somewhat less, a ratio curiously similar to that for the forms of nitrogen. In older cells, however, the ratio rises rapidly and reaches a maximum level of about 17 (15–20) on days 13–21. It finally falls when the final period of hydrolysis sets in (days 23 and 25). Comparing the different types of culture on this basis, we

again obtain a close similarity between large-celled and small-celled types of cultures which is not otherwise seen. It also appears that while the maximum tendency to form protein is characteristic of rapidly growing cells, the maximum tendency to form insoluble carbohydrate comes at a much later stage in the life of a culture. There is, in short, a tendency for the nitrogenous type of metabolism to be overpowered or masked by the carbohydrate type as growth ceases, an effect also clearly shown by the decrease in the total nitrogen as per cent of the dry weight.

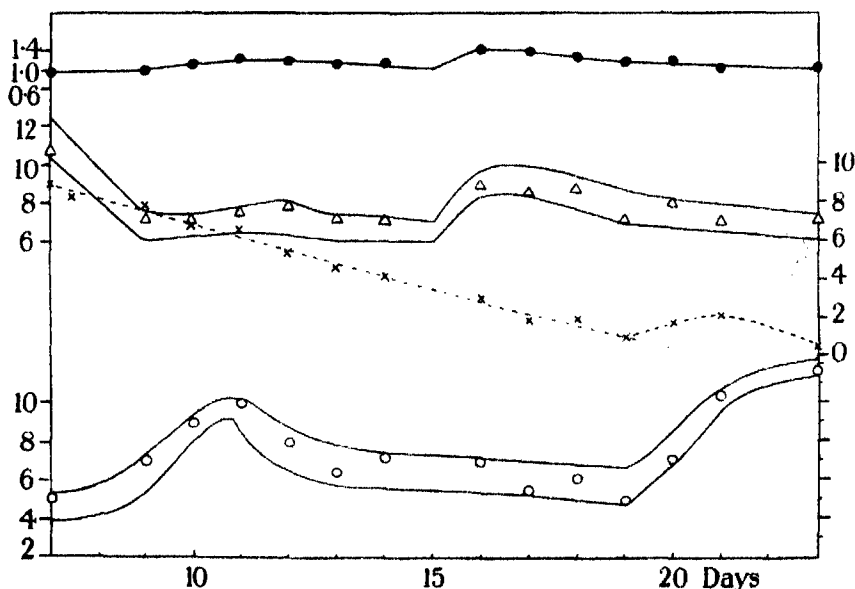


FIG. 4—Internal concentrations of soluble constituents as mg./cc. of cell volume. The curves for sugars show the range observed in check cultures. External concentrations of glucose are given for comparison, and to show the change after day 20. ● Soluble nitrogen; Δ hexose; × external glucose; ○ disaccharide.

Concentrations of Soluble Constituents

While the soluble constituents of the cells per unit protein N show fairly definite relations to the main changes going on in the cultures, it seems probable that the best method of considering these soluble constituents is to determine the *concentrations* in which they are present in the cells, since usual conceptions of metabolic changes imply that they depend on concentration. Since the dry matter present in unit volume of cells is known, we can determine the concentration of sugars or soluble N per unit of cell volume (*see p. 457*). This has been done to give the concentrations as mg. of the substance per cc. of cell volume (Table

VIII). The results, given graphically in fig. 4, prove instructive. They confirm the marked changes in the disaccharides, shown previously. The hexoses, on the other hand, fall rapidly after exponential growth ceases, and do not subsequently rise to a similar high level. Their concentration tends to remain between 6 and 9 mg./cc. in all cultures during slow growth and extension, though it may subsequently fall in old cultures, as indicated in the small-celled cultures (Table VIII, day 25). The hexoses appear to be, temporarily, at least, at a higher concentration when cell extension starts. The main feature of the hexose concentrations is, however, that they exceed 10 mg./cc. only in rapidly growing cells, this being a feature of both small- and large-celled cultures (Table VIII).

The manner in which the amounts and changes in sugar concentration agree in different cultures is a remarkable feature of the cultures, and it is in marked contrast to the differences in the concentration of soluble N. In any one culture series, the latter tends to rise during slow growth, and to be low either in rapidly growing or in old cells (*see* fig. 4). While the drift of events seems to be similar in large-celled or small-celled cultures, there are very large differences in the actual concentrations of soluble N in the two types of cultures. The small-celled cultures have roughly twice the concentration of soluble N shown by the large-celled cultures (Table VIII). It appears, therefore, that the concentration of soluble N cannot determine the growth rate, and it would appear to be possible that this rate depends largely on the concentration of hexose, which apparently must exceed 10 mg./cc. of cell for rapid growth to take place.

Insoluble Constituents

The main insoluble constituents for which data are available are protein, starch, and hydrolysable polysaccharides (including starch). The comparative changes of these constituents in relation to protein have already been considered, and it will be remembered that on that basis, the insoluble carbohydrates showed considerable irregularity, and also a marked increase after exponential growth had ceased. An alternative method of considering these fractions is to consider the gross amounts present per culture at different stages in the series (Table IX and fig. 5). This method shows that protein increases steadily until day 16 when growth ceases, although it may be noted that the *rate of increase*, as a percentage of the amount present, is declining all the time (*see* fig. 5), and more rapidly than the rates of increase of starch and total insoluble carbohydrates.

TABLE VIII—CONCENTRATIONS OF SOLUBLE CONSTITUENTS OF CELLS AND EXTERNAL MEDIUM

EXTERNAL MEDIUM					In external medium	
Day	Soluble N	Amino N	Hexose	Disaccharide	Glucose	Total N*
SERIES I						
7	0.98	0.25	10.8	5.1	9.0	2.40
9	0.97	0.28	7.1	6.65	7.9	2.04
10	1.07	0.36	7.1	8.97	6.9	1.93
11	1.22	0.44	7.5	9.8	6.65	1.82
12	1.21	0.44	8.2	8.1	5.3	1.59
13	1.15	0.36	7.2	6.45	4.7	1.49
14	1.17	0.49	7.1	7.15	4.1	1.37
16.	1.36	0.55	8.4	6.65	3.0	1.12
17	1.34	0.55	8.55	5.4	1.74	1.09
18	1.24	0.44	8.6	6.1	1.83	1.19
19	1.13	0.40	7.0	4.8	0.86	1.01
20	1.14	0.41	7.9	7.1	1.60	1.27
21	0.97	0.40	6.7	10	1.9	1.35
23	0.98	0.43	7.05	11.8	0.41	1.19
Small Cells						
X	1.88	—	12.1	4.5	10.3	2.65
Y	1.93	—	10.1	7.6	10.1	2.51
Z	1.57	—	9.95	5.3	9.2	2.46
10	2.35	—	7.2	9.1	8.8	2.09
15	2.36	—	6.8	5.7	5.3	1.56
25	1.61	—	4.45	12.2	0.47	1.74

* Taken from original nitrogen in medium—nitrogen in the cells.

TABLE IX—INSOLUBLE CONSTITUENTS AND DISACCHARIDES AS MG. PER CULTURE (SERIES I)

Day	Dry weight	Insoluble N	Starch	Insoluble carbohydrate	Disaccharide
7	23.7	0.842	3.67	12.3	0.432
9	84.4	2.53	12.6	44.9	1.97
10	103.5	3.11	16.6	56.0	3.13
11	121.4	3.64	18.9	69.65	3.51
12	175.5	4.51	28.9	108	3.87
13	193.4	4.99	33.2	129	3.48
14	216.7	5.52	40.1	138.5	4.09
16	265.2	6.59	46.6	178	3.87
17	296.2	6.57	51.3	206	4.06
18	287.0	6.34	55.75	178	4.36
19	314.5	7.02	72.6	176.5	4.16
20	286.0	6.11	61.4	180.5	5.46
21	278.0	5.84	55.7	182	9.12
23	316.7	6.55	60.1	201	11.34

In fig. 5 the results are plotted logarithmically in order to show the major changes in the percentage rates of formation. The lines are drawn for this purpose and do not necessarily imply any conclusions as to the relations with time.

A second noticeable feature of the data for starch and protein is the decrease in the gross amounts between days 19 and 21. This follows the dry weight changes. The evidence does not suggest that any parallel reduction takes place in the total insoluble carbohydrate. The two high

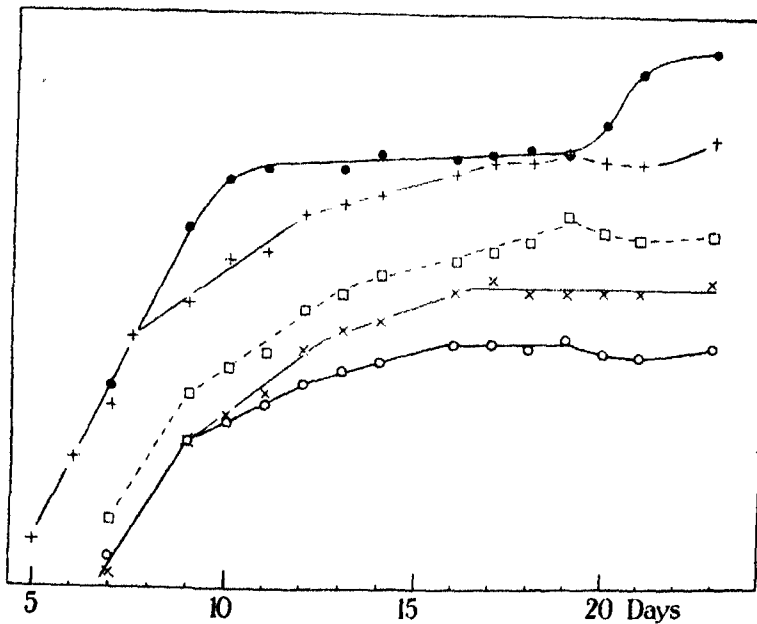


FIG. 5—Amounts of different components per culture plotted logarithmically and adjusted as to level for comparative purposes. ● Disaccharide; + glucose absorbed; □ starch; × insoluble carbohydrate; ○ protein.

values for this fraction (days 17 and 23) have not been confirmed. In five results (days 15 to 19) from a check series, for example, four of the results lay between 178 and 179 mg., the fifth was 157 mg.

In view of the apparent hydrolysis of starch after day 19, it is of interest to compare the rise in disaccharides at this time with the relative constancy of this fraction between days 11 and 19. It may be noted, further, that, during rapid growth, the logarithmic rate of formation of disaccharides apparently runs parallel with the rate of glucose absorption.

Summary of Chemical Evidence

It may be useful at this stage to summarize the chemical features of the growth cycle, so far as they are known. During exponential growth the rate of protein synthesis is very high, so much so that the formation of carbohydrates only keeps pace with it, and the proportions of protein and of insoluble carbohydrate are more equal than at any other subsequent time. The other feature apparently characteristic of this stage of growth is the high internal hexose concentration. After exponential growth has ceased, the rate of protein synthesis suddenly declines and it becomes negligible when growth finally ceases. This period of slow growth is marked by a relatively high rate of insoluble carbohydrate formation and a parallel cessation of disaccharide formation. While, however, the ratio of insoluble to soluble carbohydrates increases throughout this period, the ratio of insoluble to soluble nitrogen decreases. There is evidently a tendency towards protein hydrolysis, which becomes still more marked when the final period of cell enlargement is inaugurated. The concentrations of soluble substances tend to be high when enlargement starts. Otherwise this period is marked mainly by a definite tendency towards hydrolysis of starch, the main product being apparently disaccharides. A marked feature of this stage of hydrolysis is the visible change in the colour of the cultures. At maturity these are a deep green, but when hydrolysis becomes pronounced their colour fades until it finally becomes almost yellow or yellow-brown in old cultures. The change in colour is clearly initiated by the hydrolytic processes indicated in these analyses. It will no doubt be noticed that in many respects these changes appear to resemble those which have been demonstrated during the senescence and yellowing of leaves.

Now if we next attempt to relate the chemical data to the problem of rapid growth, the argument must be stated as follows. It has been shown that the end of the period of exponential growth is marked by a fall in the relative dry weight per cell and per unit volume of protoplasm and also by a reduction in the average size of the cells. These changes must imply that the rate of absorption of nutrient materials is falling behind the rate of growth. This, possibly, may be related to the production of smaller sized cells since the smaller size results in a higher surface-volume ratio. One might expect then that the fall in the rate of absorption would be followed by a reduction in the internal concentration of some essential nutrient. The only sign of this in the analyses is the fall in hexose concentration. It seems, therefore, that this must be the fundamental factor reducing the growth rate.

Growth must consist of a series of syntheses. We can recognize, for example, the synthesis of nucleic acid and of protein on the one hand, and, on the other, the synthesis of higher carbohydrates, such as cellulose for walls, and reserve polysaccharides and disaccharides. When the hexose concentration falls it is very probable that the rates of these different syntheses will be affected differently, and we can at least envisage the possibility that the rate of protein synthesis will decrease more than the rate of synthesis of reserve carbohydrates. The result of decreased hexose concentration would, on this assumption, be to alter the production of the cell constituents qualitatively as well as quantitatively. The data already considered (for proportions of different constituents per unit protein N) show that this is exactly what happens (*cf. also* fig. 5). When hexose concentration falls, the rate of protein synthesis evidently falls more than that of carbohydrate synthesis, so that higher carbohydrates tend to accumulate, the first to rise being disaccharide.

It is, we think, evident that there are two matters of great interest to which reference has been made, which merit more detailed attention than has been given them. These are the differences between the large-celled and small-celled cultures, and the relations between the stages of growth and the internal sugar concentrations. Both of these have been more fully investigated but, since an adequate discussion of the problems involved would diverge widely from the main aim of this paper, it has been felt advisable to curtail the evidence presented on these points. It is intended to discuss them more fully in subsequent papers.

IV—EVIDENCE FROM THE EXTERNAL MEDIUM

It is possible now to consider the relations between the cells and the glucose concentration of the external medium (*see* Tables X and XI). In doing this it should be borne in mind that the glucose estimations are accurate to 1%, so that on 400 to 500 mg. of glucose, differences need to be large to be significant. It is for this reason that it is inadvisable to discuss the question of the rate of glucose absorption in any detail, although the results do show, consistently, that the cultures absorb at least their own weight of glucose per day when growing exponentially, while after the period of exponential growth is over, the rate of glucose absorption rarely exceeds half that quantity.

The initial glucose supply in the culture medium is about 10mg./cc. There is a certain loss of sugar from caramelization when autoclaving, and in later cultures (Table XI) it was the practice to add slightly higher amounts of glucose in order to counterbalance this loss. This accounts

for the differences between Series I and Series VII and VIII. The effect of sugar concentration has been investigated in some detail, and it is known that the differences between the series under consideration do not affect any of the points raised in this paper. As the absolute amounts of glucose used during exponential growth are small and as the medium is being slightly concentrated by evaporation at the same time, the glucose concentration alters very little during exponential growth, and is about 9 mg./cc. at the end of this phase. Subsequently, however, the daily glucose losses become larger, owing to the larger size of the culture, although they are relatively less per unit of dry weight. It is a striking feature of the results that the internal carbohydrates show little or no relation to the decrease in external glucose concentration, although disaccharides show some suggestion of parallelism between days 11 and 17 (see Series I, Table VIII, fig. 4). On the other hand, it is perhaps suggestive that the internal and external hexose concentrations are similar during exponential growth (Table VIII), and the suggestion clearly is that an external glucose concentration of above 9 mg./cc. maintains inside the cells a hexose concentration suitable for rapid growth and, on the present data, more than 10 mg./cc. This seems to be a critical concentration.

A second important point evident from the data is that the sugar lost from the external medium is greater than the dry weight gain of the crop. Hence respiration losses must be greater than photosynthetic and growth gains. As the cultures are in light, this may be considered unusual, until it is reflected that the high internal concentrations of sugar may depress photosynthesis, as the end products of the latter process will be present in excess. We have unpublished data to show (a) that photosynthesis is only about 50 to 70% of the respiration under these light conditions in glucose media; (b) that the rate of respiration is only about one-third and the rate of photosynthesis is about twice as great in media without glucose. These data do not completely describe the relation of photosynthesis and respiration to the different growth phases and will not be considered here. They do, however, suggest that photosynthesis is low when sugar is supplied, and Bristol Roach (1928) has given evidence for *Scenedesmus* which further supports this assumption. It is equally probable that respiration will be larger when glucose is supplied to the cultures, even without the evidence mentioned above. For it might be inferred that the presence of glucose in the medium may tend to be associated with higher internal sugar concentrations, and hence with higher respiration rates.

If these assumptions are well founded then they should apply particularly to cells in the exponential stage of growth, for these have high internal

hexose concentrations. When, however, exponential growth ceases, the internal hexose concentration falls, and, hence, on *a priori* grounds, we might anticipate that a higher ratio of photosynthesis to respiration would become possible, and that a larger proportion of the respiratory carbon-

TABLE X—GLUCOSE USED AND REMAINING IN THE EXTERNAL SOLUTION
AS MG. PER FLASK—(SERIES I)

Day	Present	Absorbed	Crop weight	% used
0	455	0	0	—
5	445	10	3.2	32
6	433	22	7.8	35.5
7	418	37	23.7	64
7.5	385	70	32.1	46
9	359	96	84.4	87
10	310	145	103.5	71
11	296	159	121.4	76.5
12	233	222	175.5	79
13	205	250	193.4	77
14	177	278	216.7	78
16	127	328	265.2	78
17	72	383	296.2	77
18	75	380	287.0	75.5
19	39	416	314.5	75.5
20	64	391	286.0	73
21	75	380	278.0	73
23	16	439	316.7	72

Check series—large cells. Times equivalent to above

0	445	0	0	—
4.6	437	8	2.0	25
5.6	416	29	5.6	19
7.6	385	60	38.1	64
8.6	348	97	85.4	88
9.6	320	125	116.7	93
10.6	290	155	128.4	83
11.6	219	226	176.5	78
14.6	101	344	258.7	75
15.6	102	343	263.8	77
16.6	41	404	298.3	74
17.6	61	384	285.3	74
18.6	29	416	317.3	76

dioxide would be assimilated. We might, therefore, anticipate that the period of slow growth would reveal a higher proportion of dry weight gain to sugar loss, as compared with the period of exponential growth. This proportion is given in Tables X and XI as the gain in dry weight as

a percentage of the total glucose used. For the sake of brevity it may be referred to as the "percentage used" (*cf.* Terroine and Wurmser, 1922).

An examination of the tables shows very clearly that the percentage used is consistently higher for slowly growing cells than for those growing exponentially, in agreement with the argument advanced above. We conclude, therefore, that photosynthesis is *relatively* higher during slow growth. Actually, of course, it may be either that photosynthesis is more rapid or that respiration is less or that both may change. In regard

TABLE XI—GLUCOSE USED AND REMAINING IN EXTERNAL MEDIUM AS MG. PER CULTURE (SMALL-CELLED CULTURES)

	Present	Absorbed	Crop weight	% used	
Series 7	507	0	0	—	
	499	8	0.75	9.4	
	485	22	2.30	10.5	
	476	31	5.5	17.8	
	467	40	15.8	39.5	end of exponential phase A
	435	72	26.0	36	
	405	102	60.2	59	
	314	193	107.1	55	A + 6 days
Series 8	500	0	0	—	
	490	10	1.5	15	
	482	18	3.5	19	
	473	27	9.7	36	
	461	39	14.2	36.5	B end of exponential phase
	399	101	62.9	62	
	382	118	77.4	66	
	287	217	125	57.5	B + 5 days

Time intervals irregular; data from single cultures.

to the second possibility, there are two good arguments for supposing that respiration is lower in slowly growing cells. We might infer this from the lower sugar concentrations and also from the fact that growth is slower, whence the rate of energy liberation would be expected to be less. In regard to the rate of photosynthesis, there is also the possibility that the larger chloroplast surface in older and slowly growing cells (p. 476) may imply an increased rate of photosynthesis. Another point of interest in connexion with the percentage of glucose used is that the large-celled cultures (Series I) are consistently more efficient than the small-celled cultures (*cf.* Tables X and XI) and retain in the cells a larger proportion of the glucose used. We have not investigated the meaning

of this fact, but tentatively attribute it to the higher respiration rate of small-celled cultures. We hope to discuss the differences between large- and small-celled cultures more fully in a later paper.

Absorption of Ammonium Nitrate

The absorption of ammonium and nitrate ions has been followed continuously in several series, of which one is Series I. The data for this series appear to be representative, and it is proposed to limit the discussion

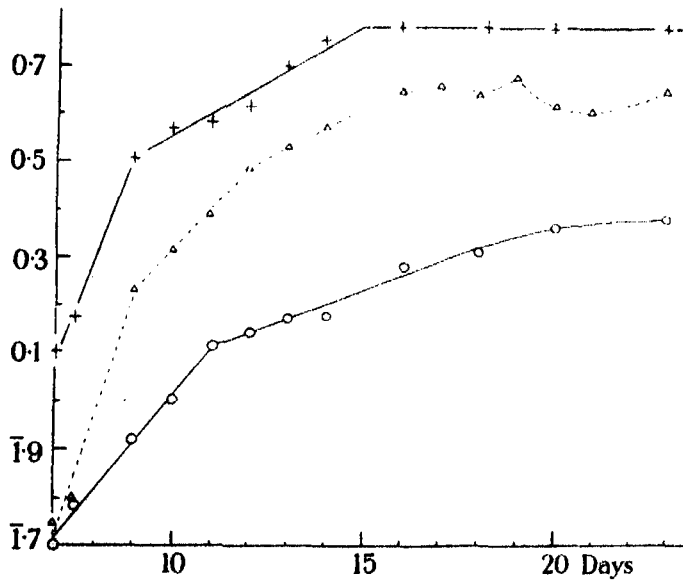


FIG. 6—Logarithms of nitrogen absorbed in different forms and of cell nitrogen in relation to time, showing the major changes in the rates of absorption of ammonia and nitrate. + ammonia N; Δ cell N; O nitrate N.

to this series of results as given in Table XII. It is noticeable that ammonia is always absorbed more rapidly than nitrate and that the nitrate remains still unexhausted at the end of the observations. Normally only about one-third of the nitrate present has been used by the time growth ceases (day 16 in Table XII). This is the more remarkable as *Chlorella* can reduce nitrate to ammonia with great rapidity, a fact shown by Warburg and Negelein (1920), and frequently confirmed for our cultures. Moreover, the absence of nitrates from the cell extracts also implies their rapid reduction in the cells. The facts serve, therefore, to suggest that nitrogen supply does not limit growth in these experiments, and it also supports the view that ammonia is preferentially used in growth.

The ammonium ions are not only absorbed more rapidly than those of nitrate, but when they are exhausted growth ceases. If the logarithm of the ammonium ions absorbed is plotted against time (fig. 6), then it appears that the logarithmic rate of absorption changes at the same times as the rate of growth, days 9 and 16. In contrast the logarithmic rate of nitrate absorption remains at its higher level until day 11, and afterwards absorption is maintained until the end of the observations. The reason for the comparatively late alteration in the rate of nitrate absorption is not clear.

Another point which arises from the consideration of these figures is that there is normally a lack of agreement between the two possible estimates of the total nitrogen absorbed. The amount of nitrogen absorbed can be estimated by the losses of ammonium and nitrate ions from the external medium. It can also be estimated (by analysis) from the known amounts of nitrogen in the cells. It will be seen from Table XII that these two estimates do not agree and that there is unaccounted for a quantity of the order of 0.5 to 1.3 mg. of nitrogen. In endeavouring to trace this quantity, we first checked the methods of estimating ammonia and nitrate (*see* Appendix). The possible error in these estimates appears to be of the order of 1%. The possible error in the estimate of nitrogen in the cells will be the product of those in estimating nitrogen and in estimating dry weight, not more than 4%. It is, therefore, apparent that the nitrogen unaccounted for must represent quite significant losses. As no routine estimate of total nitrogen in the external solution was made, it was possible that the cells had lost some organic nitrogen to the external medium. To examine this possibility, the media from some slowly growing cultures were subsequently analysed. In addition to the ammonia and nitrate present these were found to contain between 0.3 and 0.6 mg. of other nitrogen. No amino-nitrogen appeared to be present. It appears from this result, that the other (presumably organic) nitrogen in the medium is distinctly less than that lost in Series I. Bristol Roach and Page (1923) found, however, that "luxuriantly" growing cultures of *Chlorella* in sugar solutions suffered losses of nitrogen. They measured the total nitrogen of medium and culture together, and the losses encountered were of the order of 0.5 mg. Presumably this represents nitrogen lost in the elementary form to the air. We have confirmed the existence of such a loss for our type of culture in other investigations.

Taking all these facts together, we suggest that in Series I there is a loss of elementary nitrogen of about 0.5 mg., while the cultures also lose to, and may reabsorb from, the medium a similar quantity of organic nitrogen or perhaps slightly more. Thus the figure for nitrogen lost

(0.48 mg.) on day 16 represents mainly, or entirely, losses of elementary nitrogen. The figures of 1.0 mg. or upwards at other times represent organic nitrogen escaped to the culture medium, in addition to the elementary nitrogen lost. It is obvious, if this suggestion is correct, that the cells must be periodically reabsorbing and losing organic nitrogen. In this respect, the rise of "escaped nitrogen" from day 16 to day 20 (0.86 mg.) appears to be quite significant, and it is of interest to note that it is correlated with a fall in the concentration of soluble nitrogen inside the cells (Table VIII). It is also of interest to note that the external

TABLE XII—NITROGEN ABSORBED FROM EXTERNAL SOLUTION AS MG. PER FLASK—(SERIES I)

Days	In external medium		Total N absorbed	Total N in cells	N unaccounted for	Final pH
	Ammonia N	Nitrate N				
0	6.1	6.0	—	—	—	6.00
7	5.0	5.5	1.6	0.93	0.67	5.87
7.5	4.6	5.4	2.1	1.08	1.02	—
9	2.9	5.2	4.0	2.83	1.17	5.77
10	2.4	5.0	4.7	3.42	1.28	5.72
11	2.2	4.7	5.2	4.08	1.12	5.60
12	2.0	4.6	5.5	5.08	0.42	5.50
13	1.1	4.5	6.5	5.61	0.89	5.50
14	0.4	4.5	7.2	6.21	1.0	5.32
16	0.1	4.1	7.9	7.42	0.48	5.18
18	0	3.95	8.15	7.23	0.92	5.01
20	0.04	3.70	8.36	7.02	1.34	4.81
23	0.04	3.6	8.46	7.51	0.95	4.78

sugar concentration apparently rose at the same time (days 19–20, Table VIII, fig. 4), and there is, further, a rough general correlation between times when the glucose absorption was rapid and when the nitrogen loss fell to a low level (for example, days 11 and 12). It is obviously probable that cultures containing dividing and dying cells may lose some organic substances to the external medium which may subsequently be reabsorbed. Attention has already been drawn to the fluctuations of dry weight in the older cultures and the view expressed that these are largely significant (p. 461). A small dry weight, such as occurs on day 20, would then represent a period following the death of a part of the cell population, when their soluble constituents would be present in the external medium. It is evident that the data agree consistently with the explanation advanced, though we have no wish to over-emphasize it or to examine it here in

great detail, since the method of replicate cultures used is far from being the most suitable one for studying such changes.

SUMMARY AND CONCLUSIONS

The observations recorded show that when *Chlorella* is grown in a limited quantity of a glucose nutrient solution under the named conditions, growth proceeds exponentially until the cell number reaches 6000 cells per cu. mm. After this growth proceeds at a lower rate until the cell number is about four to five times as great. Increase in cell number is then replaced by cell extension.

The exponential period of growth is mainly characterized by declining cell size and decreasing dry weight per cell, though these properties appear to increase during a short initial period. The dry weight per unit volume of cell material also appears to be minimal at, or just after the end of, exponential growth. Hence the average rate of increase of cell number tends to be higher than that of culture volume and the rate of increase of culture dry weight is usually less than that for culture volume. There are signs, indeed, that these growth rates may vary at different times during the period of exponential growth.

The changes in size and in relative dry weight which occur during growth are paralleled by successive changes in the appearance and structure of the cells. In general, these changes are in the direction of increase in vacuolation, in chlorophyll and starch content, and in wall thickness. At the same time, the method of cell division also becomes modified, without, however, affecting to any significant extent the logarithmic growth rate.

The evidence from microscopic examination thus suggests that cell metabolism changes from a form involving mainly protein and protoplasmic synthesis to one in which wall and carbohydrate accumulation predominates. This suggestion is borne out by chemical analyses, which show that when growth slows down the rate of protein synthesis decreases more than the rate of formation of higher carbohydrates, so that the latter tend to accumulate relatively more rapidly. The cause of this change appears to be a fall in the internal concentration of hexoses, which develops very rapidly at the end of the period of exponential growth. About a week after growth has ceased, a general hydrolysis of insoluble materials sets in and the cultures then tend to become yellow in colour. Accumulation of disaccharides is noticeable at this time, and significant amounts of organic substances may escape to the external medium. Some of these may be reabsorbed.

These changes markedly resemble those observed in senescent and yellowing leaves. The structural changes in the cells during growth also run parallel to those characteristic of cells developing in the meristems of higher plants. There is, therefore, a striking resemblance between the developmental and maturation changes in algal cells such as these and the parallel stages in cell development in higher plants.

APPENDIX

Analytical Methods—The cultures were prepared for analysis by filtering them on to a well-washed asbestos pad in a Gooch crucible. This was sucked dry, heated to 95° C. for half an hour, to destroy enzymes, and then dried to constant weight at 65° C. This method has been shown to be appropriate for green tissues by Link and Tottingham (1928) and Pearsall and Wright (1930). The dry material was stored for analysis in desiccators. It forms a hard pad on the surface of the asbestos. This pad is removed with as little asbestos as possible, carefully weighed, and ground up to a fine powder for analysis. It contains some asbestos and the proportion of the latter is estimated from the following weighings taken:—

1. Crucible + asbestos.
2. Crucible + asbestos + dry *Chlorella*.
3. Crucible + asbestos after removal of *Chlorella*.
4. *Chlorella* removed + any asbestos taken with it.

From these we get:—

(a) Weight of *Chlorella* is $2 - 1$.

(b) Weight of *Chlorella* + asbestos removed (4) should equal $2 - 3$.

From (a) and (b) the percentage of *Chlorella* or asbestos in the analytical material can be calculated. This material normally contained between 95 and 98% of *Chlorella*.

About 200 mg. of this powder was extracted in 60% alcohol (1 cc. to 4 mg.) for 24 hours at room temperature, all material in any one series of cultures being extracted at the same time. This method precipitates typical plant proteins and gives complete extraction of soluble material (Pearsall and Wright, 1930). Amino-acids and titratable acidity were estimated within twenty-four hours on triplicate fractions (normally 5 cc.) of this extract by Sørensen's formol titration method, using N/100 acid, a micro-burette, and a comparator technique like that of Cole (1928). The titrations, though small, were exactly reproducible.

Soluble nitrogen was estimated in triplicate on aliquots (10 cc.) containing about 0.1 mg. of nitrogen. These were evaporated to dryness before digestion. The micro-Kjeldahl method of Pregl (1930) was employed. The replicates normally agree to within 0.004 mg. of nitrogen, that is, one drop of N/100 alkali from the micro-burette used. The error of the mean should not be more than 1%, the figure obtained from check estimations on prepared solutions of known nitrogen content.

Reducing substances were estimated in triplicate on 2 cc. of the extract by the method of Hagedoorn and Jensen (1923). Hane's modification of this method (1929) was used for estimations of glucose in the external medium. Disaccharides in the extract diluted five times were hydrolysed by heating in boiling water with a similar volume of normal hydrochloric acid for ten minutes, followed by exact neutralization. In known sugar solutions the sugars could be estimated to within 1%. Starch was estimated as glucose on about 20 mg. of original powder, boiled in water, cooled, and then incubated with taka-diastrase (1 enzyme: 20 material) at 35° C. for three days. Total hydrolysable polysaccharides were estimated on a similar quantity of powder as glucose, after hydrolysis with 3% sulphuric acid for three hours at 100° C. under a reflux condenser. The extract was filtered and neutralized to p_H 7 with sodium carbonate. The insoluble carbohydrate estimations were made in duplicate. The mean of these duplicates may vary by 3% when a series of repetitions is carried out on a single sample. In regard to the starch, however, although an accuracy of this order could be obtained on starch solutions, allowing for the presence of maltose, much greater difficulty was obtained with *Chlorella* material in obtaining agreement between duplicates or check determinations. We think an error of 10% in any individual estimate given is possible, although the general drift of the starch variations is quite definite and can readily be checked. We omit this determination as a routine measure in our later analyses, but include one series of results (repeated twice) for information.

Total nitrogen was estimated by the micro-method of Pregl (1930) in four replicates, each of about 10 mg. of powder. The variation of the mean from the probable value is less than 1%, as checked by successive determinations on the same powder and on powders of known nitrogen content. The insoluble or protein nitrogen is obtained by difference, and as the soluble nitrogen is only one-eleventh of the total nitrogen, the error in the estimate of protein nitrogen will be mainly that of the total nitrogen. We assume accordingly that the estimate of protein nitrogen lies within 2% of the probable value.

In all estimations of nitrogen N/100 hydrochloric acid was used to

receive ammonia, and the back titration carried out (after boiling) with N/100 sodium hydroxide from a micro-burette using methyl red as indicator.

The minimum weight required for these analyses in full is about 300 mg. By reducing the total N replicates to three, omitting starch and amino N, the analyses can be carried out on 150 mg. The micro-methods employed will all deal with much smaller quantities than those used, but as the experimental error of the determination does not usually decrease much, the standard of accuracy does not usually remain as high when small quantities are analysed.

TABLE XIII—CARBOHYDRATE FRACTIONS AS PER CENT OF THE DRY WEIGHT

SERIES I				
Day	Hexose	Disaccharide	Starch	Total insoluble carbohydrate
7	3.85	1.83	15.5	52.1
9	2.51	2.34	15.3	53.3
10	2.40	3.02	16.0	53.8
11	2.21	2.89	15.6	57.4
12	2.23	2.21	16.5	61.6
13	2.01	1.80	17.2	66.7
14	1.87	1.89	18.5	63.8
16	1.85	1.46	17.6	67.2
17	2.16	1.37	17.3	69.6
18	2.16	1.52	19.4	61.9
19	1.92	1.32	23.1	56.1
20	2.15	1.91	21.5	63.1
21	2.20	3.28	20.5	65.3
23	2.15	3.58	19.0	63.3
<i>Small Cells</i>				
X	2.96	1.10	17.6	40.5
Y	2.37	1.78	16.5	39.9
Z	2.54	1.35	16.6	35.5
10	1.38	1.74	—	42.6
15	1.30	1.09	—	46.8
25	0.99	2.72	—	32.0

The external solution after the removal of the cells was stored under toluol for analysis. Before this, however, the hydrogen ion concentration was determined electrometrically, using quinhydrone and calomel electrodes, checked against standard phosphate buffer solutions. The

solution was subsequently analysed for sugar content (as above), and for ammonia and nitrate nitrogen. After making the solution alkaline, ammonia was removed by steam distillation into N/100 acid (as above). Devarda's alloy was then added and the ammonia formed from nitrate reduction was estimated after an hour. Four replicates were used and the mean taken. From check determinations on solutions of known ammonium nitrate concentration, it appears that the mean determinations are within 1% of the probable value.

TABLE XIV—NITROGEN FRACTIONS AS PER CENT OF THE DRY WEIGHT

Day	Total N	SERIES I		
		Insoluble N*	Soluble N	Amino N
7	3.91	3.56	0.35	0.09
7.5	3.37	—	—	—
9	3.35	3.00	0.35	0.10
10	3.36	3.00	0.36	0.12
11	3.36	3.00	0.36	0.13
12	2.90	2.57	0.33	0.12
13	2.90	2.58	0.32	0.10
14	2.86	2.55	0.31	0.13
16	2.79	2.49	0.30	0.12
17	2.56	2.22	0.34	0.14
18	2.52	2.21	0.31	0.11
19	2.54	2.23	0.31	0.11
20	2.45	2.14	0.31	0.11
21	2.42	2.10	0.32	0.13
23	2.37	2.07	0.30	0.13
<i>Small Cells</i>				
X	4.85	4.39	0.46	—
Y	4.87	4.42	0.45	—
Z	4.29	3.89	0.40	—
10	4.25	3.80	0.45	—
15	3.59	3.14	0.43	—
25	2.57	2.21	0.36	—

* By difference.

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Inhibition of Action of Pituitary Hormones by Animal Sera*

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[PLATE 16]

INTRODUCTION

In these 'Proceedings' for 1934 (Thompson and Cushing, 1934) appeared the report of an experimental effort to reproduce the clinical syndrome of pituitary basophilism in a puppy by means of injections of a gonadotropic extract prepared from sheep-pituitaries. Certain striking effects were observed in the experimental animal, namely, obesity, a lowered metabolism, sexual inactivity, and retardation of growth with skeletal dystrophy. These effects, while in some respects suggesting the human disorder, basophilism, could in others be interpreted broadly as an inhibition of normal processes simulating hypophysectomy.

Further experiments with other gonadotropic preparations have since been made, and each time some form of inhibition has developed in the animals. In order to investigate the nature of these reactions the blood sera of some of the injected dogs were tested by methods suggested by

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Collip and his co-workers in their several reports concerning "anti-hormones" (Anderson and Collip, 1934, *a, b, c*; Bachman, 1935; Bachman and Collip, 1934; Collip, 1934; Collip and Anderson, 1935). It will be recalled, briefly, that Anderson and Collip (1934, *a*) first detected an antagonistic activity of the blood sera of animals which had become insensitive to injected thyrotropic extract. Bachman, Collip, and Selye (1934) shortly afterwards stated in regard to gonadotropic extract that "the loss of sensitivity is limited to the gonadotropic preparation with which the animals have been injected previously. Animals which become insensitive to pituitary implants remain sensitive to A.P.L.* and *vice versa*". The term "antihormone", subsequently used by Collip and his co-workers to indicate this inhibitory action, is now well known and requires no explanation.

Since the exact nature of this phenomenon has not yet been disclosed, the terms "antagonistic principle" or "inhibitory action" will be used in the present report, which will cover our own experiments in a similar direction with the sera of animals rendered resistant to gonadotropic hormones.

The effect in treated animals† appears to have been produced by injections of extracts of glands from species of animals different from those receiving the injections. This fact immediately suggested the possibility of the presence of an antigenic substance in the preparation. Working on this assumption, Bachman (1935), of Collip's laboratory, briefly reported an unsuccessful attempt to detect with the complement fixation test a connexion between A.P.L. as the antigen and the serum of rabbits rendered resistant to it by continued injections.

Twombly (1936) extended the immunologic studies of A.P.L. and showed: (1) that precipitins can be found in the sera of rabbits immunized with this hormone preparation; (2) that partially inactivated extracts are almost as efficient as the active preparations in bringing about the formation of protective responses; and (3) that in the sera of three patients to whom the hormone had been given for a considerable time no immune responses or antagonistic effects were demonstrable.

On the other hand, as Twombly intimates, neither P. E. Smith's original implantation experiments nor the more recent studies of para-

* Anterior pituitary-like gonadotropic hormone of the urine of pregnancy (Collip), more commonly known as prolan.

† Eitel and Loeser (1934) have found that the sera of untreated sheep have an antagonistic action against thyrotropic hormone. This proved true in one instance at our hands when we tested lamb's serum in guinea-pigs and found that it inhibited a substantial dose of sheep thyrotropic hormone.

biosis (Du Shane, Levine, Pfeiffer, and Witschi, 1935; McCahey, Solway, and Hansen, 1936) have led to anything that could be interpreted as involving the production in the animals of an antagonistic or inhibitory process. The first reports of failure to produce antagonistic effects of this sort by injections of extracts from animals of other species were made by Werner (1936, *a*, *b*), who found that prolonged injections of a beef-pituitary flavianate containing thyrotropic hormone did not lead to the production of inhibition in guinea-pigs, and, likewise, small doses did not lead to an immunity against it as was true of Twombly's prolan preparation. Werner's findings would seem to indicate: (1) that a possible hormone-antigen complex had been disrupted by the flavianic acid procedures, with inactivation or separation of the antigenic portion, or, that (2) a true hormone with anti-thyrotropic qualities which might have been present in the original preparation had been removed.

STATEMENT OF THE PROBLEM

The facts cited above suggest that for successful substitution therapy the separation of a pituitary hormone from its possible antigenic-complex or its inhibitory cohormone is a step of great importance; no less important, before undertaking any form of counter therapy, would it be to determine whether an antiserum produced by the injection of pituitary extract of one species of animal can prevent the action of pituitary hormones from another species. The question may be stated as follows: *What evidence can be produced to show whether injection into a dog of extracts from sheep pituitaries will produce an antiserum which will inhibit the action of pituitary hormones prepared from species other than sheep or dog?*

Our first tests showed that the serum of one dog after prolonged injections with a sheep pituitary gonadotropic extract had acquired strong inhibitory powers against the gonadotropic action of that extract. The serum was then injected into immature rats in which what seemed to be purely inhibitory effects were observed without other complicating factors. The normal sexual development of these animals was checked by the injections of the antiserum whose effects simulated hypophysectomy, so far as the gonadotropic functions were concerned.

The inhibition of reproductive development of the young rat might in these tests have been due to an inactivation of its own gonadotropic hormones produced by the antiserum. This suggested that the antiserum was not species specific since the gonadotropic principles of both dog and rat were depressed below normal. Results of this sort for two

species do not establish the non-specificity of the antagonistic action and cannot be construed as applicable to man. Further experiments with pituitary hormones from other species including man were then undertaken, with the results to be described.

THE SEVERAL HORMONE PREPARATIONS AND SERA EMPLOYED IN THE EXPERIMENTS

Extract No. 1—This preparation, used by us in our original experiment (Thompson and Cushing, 1934), was essentially that described by Van Dyke and Wallen-Lawrence (1933). For convenience it is designated "Van. D. and W.-L extract". Fresh whole sheep-pituitaries, ground in a mortar with fine sand, are extracted with 0.02/N ammonia for eight hours, after which the p_H is adjusted to 5.4 with hydrochloric acid and sodium acetate-acetic acid buffer. The material is then centrifuged, and 95% alcohol is added to the supernatant fluid until the concentration is 80%. The precipitate which forms is taken up in distilled water, centrifuged, and alcohol is again added to the supernatant fluid until the concentration is 80%. The precipitate formed is then dissolved in sufficient sterile Ringer's solution so that 3 cc. of extract are obtained from one sheep-pituitary gland. When prepared in this way, large amounts can be injected subcutaneously without causing induration of the tissues.

This method of preparation differs from that of Van Dyke and Wallen-Lawrence in that (1) we use hydrochloric acid instead of glacial acetic acid for neutralization of the ammonia; (2) the hormone is precipitated twice with 80% alcohol instead of 70% after a preliminary treatment with 35% alcohol; and (3) the final extract is taken up in sterile Ringer's solution with sterile technique so that Berkefeld filtration or the use of preservative is not necessary.

Extract No. 2—Another sheep-preparation was made from a stock of acetone-dried powder of fresh sheep-pituitaries kindly supplied by Dr. H. M. Evans. At intervals small amounts of the powder were extracted according to his directions with 0.02/N NaOH for eight hours, after which time the fluid was brought to a p_H of 7.4 with 0.1/N hydrochloric acid. It was then centrifuged, and the supernatant fluid with merthiolate 1:10,000 as preservative was the extract employed. From 1 gm. of powder was prepared 100 cc. of extract. Considerable subcutaneous induration developed at the site of each injection of this material.*

* Mentioned because of the possible relationship to the formation of immune bodies. This applies also to extract No. 5.

Extract No. 3—A third sheep-pituitary gonadotropic preparation, obtained through the cooperation of Dr. J. B. Collip, was used for testing the antisera. It was not injected for long periods into animals.

Extract No. 4—Two gonadotropic preparations of hog-pituitaries were obtained through the courtesy of Parke, Davis & Company. They were labelled "Antuitrin, gonadotropic, No. 095813A and No. 351129BD1".

Extract No. 5—A highly active gonadotropic extract of the serum of pregnant mares, prepared in powder form by the method of Catchpole and Lyons (1934), was also kindly supplied by Dr. H. M. Evans. The powder was dissolved in 0.02/N sodium hydroxide, and the p_H was brought to 7.4 with 0.1/N hydrochloric acid after merthiolate 1:10,000 had been added. 1 cc. of extract equalled 0.01 gm. of powder. A fox-terrier puppy, whose serum was studied, received injections of 25 cc. of this material daily for four months. This extract also caused marked induration at each site of injection.

Extract No. 6—A gonadotropic extract of human urine of the menopause, "probably of pituitary origin", was generously contributed by Dr. E. T. Engle and Dr. Louis Levin.

Extract No. 7—The hormone of the urine of pregnant women was supplied by Parke, Davis & Company. The samples were marked "Antuitrin S No. 0961712".

The Sera.—These were prepared from blood obtained by venipuncture with sterile precautions so that no preservative was needed. A fresh supply was taken at intervals of at least one week.

TESTS EMPLOYED

The gonadotropic activity of each extract was determined by a standard hundred-hour test in immature female rats twenty-three to twenty-five days old. Three injections of the extract were given subcutaneously daily for two days, and the animals were sacrificed one hundred hours after the first injection. At autopsy the appearance of introitus and uterus were noted, and the ovaries were weighed, these weights serving as indication of the degree of activity of the extract.

The power of a serum to inhibit the action of these standardized gonadotropic preparations was determined by injecting another group of rats with serum twice daily beginning two days before the hundred-hour period and continuing through the hundred-hour period. The

object was to have the serum in the process of absorption during the action of the injected gonadotropic hormones.

Ovarian weights cited in the experiments represent the average weight of ovaries of at least four tested rats, unless otherwise designated.* In some of the tests of the puppy sera fewer rats were injected because of the limited quantity of serum.

THE ANIMALS PROVIDING THE TESTED SERA

1—A large female collie dog ("Alice") was the source of the most potent antiserum. She had been injected daily with 25 cc. of the Van D. and W.-L. extract (No. 1) for seventeen months, during which time the animal became obese and showed marked hypercholesterolemia together with inhibition of sexual functions.

2—One female fox-terrier puppy, three months old at the start of the experiment, was injected daily with 25 cc. of the Evans sheep-pituitary extract (No. 2) for four months. The puppy matured rapidly and showed continuous marked sexual stimulation. However, it resembled a hypothyroid animal in its sluggishness and in the coarseness of its fur. The cholesterol values of the blood proved to be normal, in spite of the fact that the thyroid gland was found at biopsy and autopsy to be inactivated.

3—Another female fox-terrier puppy, a litter-mate sister of animal No. 2, was injected daily for four months with 25 cc. of the gonadotropic extract of the serum of pregnant mares (extract No. 5). This puppy showed early sexual stimulation which rapidly regressed. The animal's teeth developed poorly, and the hair became fluffy like the fine fur of young hypophysectomized rats.

4—Two normal female and two normal male dogs which had no injections served as controls.

RESULTS OF THE TESTS

1—*The Serum of the Collie Dog, "Alice"*

The studies of this serum were devised so as to determine whether the action of the several gonadotropic hormones already described could be checked. In order to secure definite results, substantial doses of the serum and extracts were used. The tests showed that the serum inhibited the gonadotropic action of each extract so effectively that in every animal

* Starred numbers in Tables IV and V indicate that only two instead of the customary four animals were used in the tests.

that received injections of both serum and extract the uterus and ovaries showed no signs of activation and the introitus remained closed. The data are recorded in Table I, and are illustrated by figs. 1, 2, and fig. 3, Plate 16.

TABLE I—INHIBITORY INFLUENCE IN RATS OF COLLIE DOG'S SERUM UPON THE GONADOTROPIC ACTION OF THE SEVERAL HORMONE PREPARATIONS

Source of gonadotropic hormone	Dose of extract (cc.)	Ovarian weights (mg.)	
		100-hour test extract alone	100-hour test extract plus serum 0.5 cc. doses
A—Pituitary—			
Extract No. 1—Sheep; whole gland (Van D. and W.-L.)	0.5	51	11
Extract No. 2—Sheep; whole gland (Evans)	0.5	42	10
Extract No. 3—Sheep; whole gland (Collip)	0.5	86	12
Extract No. 4—Hog; anterior lobe (P. D. & Co.)—			
No. 095813A	0.5	104	24
No. 351129BD1	0.5	55	11
B—? Pituitary—			
Extract No. 5—Mare; serum of pregnancy (Evans)	0.5	179	16
Extract No. 6—Human; urine of menopause (Engle)	1.5	62	11
Extract No. 7—Human; urine of pregnancy (P. D. & Co.)	0.5	33	19

The effect of the serum upon the gonadotropic functions of the test animals themselves was observed by giving injections of 0.5 cc. of serum twice daily for fifty-six days to each of a group of five female rats starting when they were twenty-five days old. The introitus of each of the five litter-mate sister controls, which had not been injected, had opened within the following thirty days, while the introitus of each of the injected animals remained closed. On the fifty-sixth day of injections, when it seemed probable that the eighty-one-day-old injected animals would not mature sexually, the two groups of rats were sacrificed. Each control was sexually matured with open introitus, well-developed uterus, and active ovaries, while the injected animals resembled rats hypophysectomized

before maturity, having a shiny, closed introitus, a small, pale uterus, and inactive ovaries. The average weight of the ovaries of the controls was 42 mg.; that of the injected animals was 14 mg. (fig. 4, Plate 16).

Smaller doses of serum than were used in the foregoing experiments served materially to check standard doses of the Evans sheep-pituitary preparation. The effects of increasing doses of the serum upon a standard amount of gonadotropic hormone are shown in Table II.

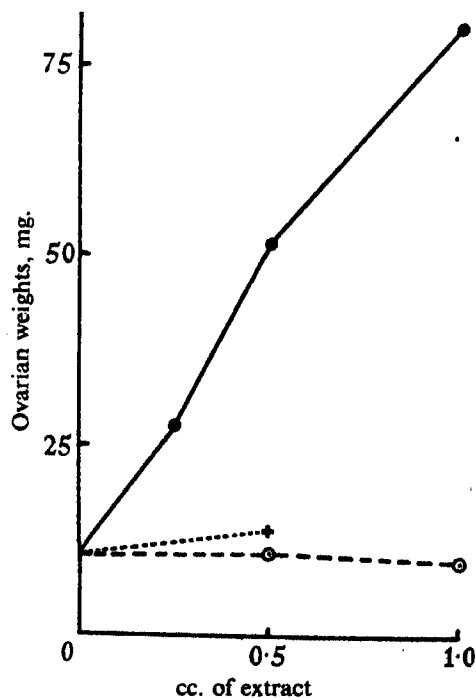


FIG. 1—The effect on ovarian weights in rats of increasing doses of the gonadotropic hormone of the Van D. and W.-L. extract as compared with the effect of this same extract plus the serum of the collie dog. ● After increasing doses of the extract; ○ after the same amounts of extract plus 0.5 cc. doses of the serum; + after the same amounts of extract plus 0.1 cc. doses of the serum.

One experiment was performed in which only a single injection of 0.5 cc. of serum was given each test animal at the time of the first injection of the gonadotropic hormone in the hundred-hour test. This single dose of serum was quite effective in inhibiting the Van D. and W.-L. extract, as shown in Table III.

2—The Serum of the Puppy Receiving the Evans Sheep-Pituitary Extract

This serum gave different results from those obtained with the collie dog serum (fig. 4, Plate 16). In this instance the action of the extract

TABLE II—THE EFFECT OF INCREASING DOSES OF THE INHIBITORY SERUM (COLLIE DOG) UPON THE GONADOTROPIC ACTION IN RATS OF STANDARD DOSES OF THE EVANS SHEEP-PITUITARY EXTRACT (EXTRACT No. 2)

Test	Dose (cc.)		Ovarian weights (mg.)
	Serum	Extract	
Extract No. 2 alone	None	0.5	42
Extract No. 2 plus serum	0.05	0.5	18
" "	0.10	0.5	12
" "	0.3	0.5	11
" "	0.5	0.5	9

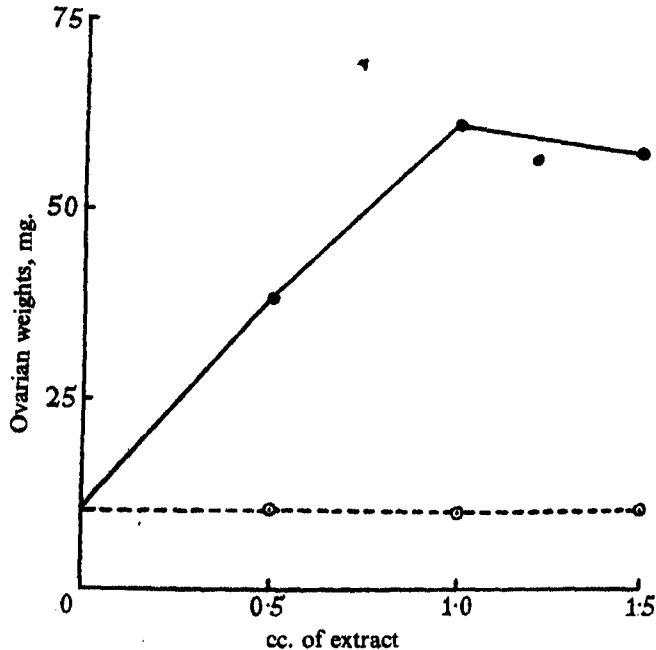


FIG. 2—The effect on ovarian weights in rats of increasing doses of the gonadotropic hormone of human urine of the menopause as compared with the effects of this same extract plus the serum of the collie dog. ● After increasing doses of the extract; ○ after the same amounts of extract plus 0.5 cc. doses of the serum.

injected into the puppy was almost completely checked in rats by the puppy's serum, but the gonadotropic preparations from other sources were not inhibited. In fact, their effects may have been somewhat augmented. The results are shown in Table IV and fig. 5.

TABLE III—THE EFFECT OF A SINGLE INJECTION OF COLLIE DOG SERUM UPON THE GONADOTROPIC ACTION IN RATS OF TWO DIFFERENT DOSES OF THE VAN D. AND W.-L. EXTRACT (EXTRACT NO. 1)

Test	Dose (cc.)		Ovarian weights (mg.)
	Serum	Extract	
Extract No. 1 alone	None	0.5	51
" "	None	1.0	73
Extract No. 1 plus one inj. serum ..	0.5	0.5	18
" " " ..	0.5	1.0	18

TABLE IV—INFLUENCE OF PUPPY SERUM (ANIMAL NO. 2) UPON STANDARDIZED DOSES IN RATS OF SEVERAL GONADOTROPIC HORMONES

Source of gonadotropic hormone	Dose of extract (cc.)	Ovarian weights (mg.)	
		100-hour test extract alone	100-hour test extract plus serum 0.5 cc. doses
A—Pituitary—			
Extract No. 1—Sheep; whole gland (Van D. and W.-L.)	0.5	51	66*
Extract No. 2—Sheep; whole gland (Evans)	0.3	30	20
" " " "	0.5	42	27
B—? Pituitary—			
Extract No. 5—Mare; serum of pregnancy (Evans)	0.25	145	204*
" " " "	0.5	179	209*

3—The Serum of the Puppy Receiving the Extract of the Serum of Pregnant Mares

The serum of this puppy proved to be partially effective in preventing the action of gonadotropic hormones of different species. The antagonistic power was less potent than that of the serum of the collie dog (fig. 6). The results are shown in Table V and fig. 6.

4—The Sera of the Controls

The control sera were tested in the same manner as the antisera for a possible power to inhibit the Van D. and W.-L. extract in standard hundred-hour tests. No definite inhibitory effect could be demon-

strated by this method. After injections of each of the control sera and the hormone preparation the average ovarian weights and appearance of uterus of the tested rats varied somewhat, but each of the tested rats had definite ovarian activity of varying degree and an open introitus. The ovarian weights are recorded in Table VI.

The effect of normal-dog serum upon the development of the reproductive system of the test animals themselves was also studied in the same

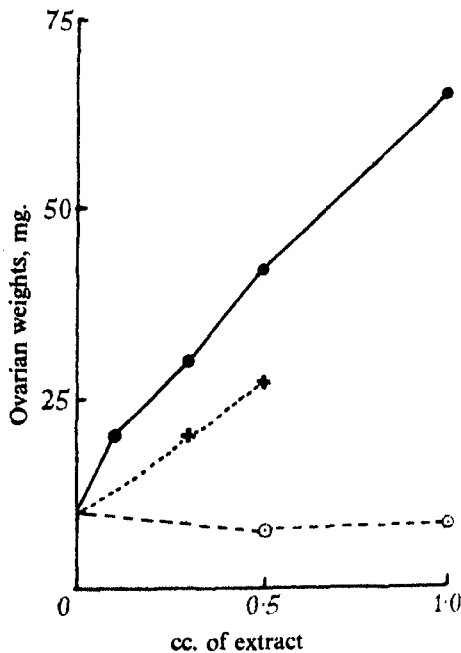


FIG. 5—The effect on ovarian weights in rats of increasing doses of the gonadotropic hormone of the Evans sheep-pituitary extract as compared with the effects of the same extract plus (1) the serum of the puppy that received injections of this same extract, and (2) the serum of the collie dog. ● After increasing doses of the extract; + after the same amounts of extract plus 0.5 cc. doses of the puppy serum; ○ after the same amounts of extract plus 0.5 cc. doses of the collie dog serum.

manner as for the serum of the collie dog. In this experiment twenty female rats were divided into three groups with litter-mate sisters represented in each group, and each of the rats was inspected daily in order to detect the date of opening of the introitus. The five rats of the first group each received injections twice daily of 0.5 cc. of the serum of a normal female dog, and each of the five rats of the second group received the same amount of the serum of a normal male dog. The third group (ten rats) received no injections. The average age at opening of the introitus

of the group that received the serum of the normal female dog was forty-seven days (youngest, forty-five; oldest, fifty-one); of the group that received the serum of the normal male dog, forty-four days (youngest, thirty-six; oldest, fifty); and of the group that received no serum, forty-seven days (youngest, thirty-two; oldest, fifty-nine). It was concluded that the sera of these two normal dogs did not retard the development of the immature rats.

TABLE V—INFLUENCE OF PUPPY SERUM (ANIMAL NO. 3) UPON STANDARDIZED DOSES IN RATS OF THE SEVERAL GONADOTROPIC HORMONES

Source of gonadotropic hormone	Dose of extract (cc.)	Ovarian weights (mg.)	
		100-hour test extract alone	100-hour test extract plus serum 0.5 cc. doses
A—Pituitary—			
Extract No. 1—Sheep; whole gland (Van D. and W.-L.)	0.5	51	93
Extract No. 2—Sheep; whole gland (Evans)	0.5	42	27*
Extract No. 4—Hog; anterior lobe (P. D. & Co.) No. 095813A	0.5	104	82*
B—? Pituitary—			
Extract No. 5—Mare; serum of pregnancy (Evans)	0.25	145	13*
" " " "	0.5	179	93*
Extract No. 7—Human; urine of pregnancy (P. D. & Co.)	0.5	33	26*

DISCUSSION

These experiments provide evidence that injections of an extract (No. 1) of sheep pituitary glands aroused in the collie dog's serum an inhibitory principle which had the power of checking the action of the gonadotropic hormones of widely different species, including man. The antagonism was highly effective, shown by the fact that doses of 0.05 cc. of antiserum administered twice daily materially inhibited a standard dose of hormone. The effect of the antiserum appeared to be equally marked for all gonadotropic extracts tested, irrespective of source, *i.e.*, species or method of preparation.

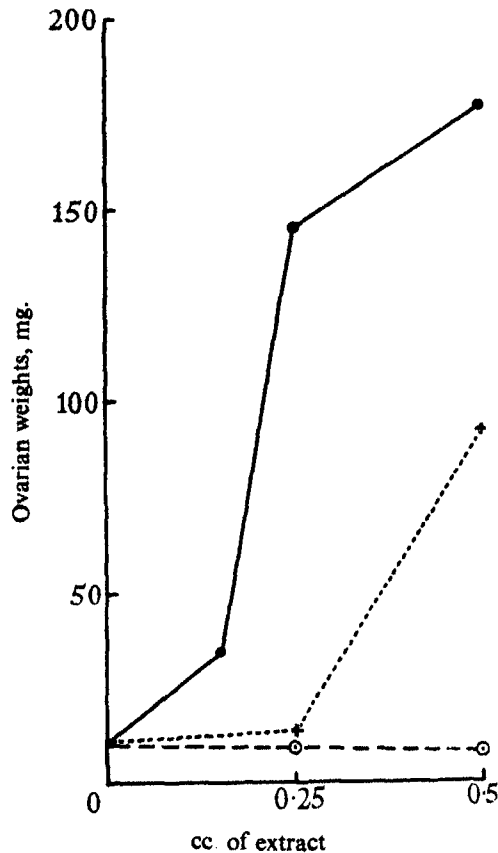


FIG. 6—The effect on ovarian weights in rats of increasing doses of the gonadotropic hormone of the serum of pregnant mares as compared with the effects of the same extract plus (1) the serum of the puppy that received injections of this same extract, and (2) the serum of the collie dog. ● After increasing doses of the extract; + after the same amounts of the extract plus 0.5 cc. doses of the puppy serum; ○ after the same amounts of the extract plus 0.5 cc. doses of the collie dog serum.

TABLE VI—EFFECT OF 0.5 CC. DOSES OF CONTROL SERA UPON GONADOTROPIC ACTION IN RATS OF THE VAN D. AND W.-L. EXTRACT

Test	Ovarian weights (mg.)	
	Extract 0.25 cc.	Extract 0.5 cc.
Extract No. 1 without serum	28	53
" plus serum shepherd female	35	39
" " Airedale female	30	40
" " tan mongrel male	37	43
" " terrier male	34	54

The serum of the collie dog also prevented the normal development of the reproductive system of young rats when it was injected daily during the developmental period.

Injections in a puppy of a gonadotropic extract (No. 5) of pregnant mare serum also produced a non-species-specific inhibition of the tested gonadotropic hormones. The degree of inhibition, however, produced by this serum in the standard tests was less pronounced for any of the hormones than was that of the collie dog serum. The lack of specificity of the antagonistic principle of this serum and the similarity of its action with the collie dog's serum suggest that the gonadotropic hormones of normal sheep-pituitary glands and the serum of pregnant mares are much alike in composition, since the inhibitory substances aroused by them act similarly upon gonadotropic hormones of various sources.

Should the gonadotropic hormone of the pregnant mare serum arise from the chorionic epithelial cells, as intimated by Catchpole and Lyons (1934), then it would appear that this hormone either is closely related structurally to one of the pituitary gonadotropic hormones or in the mare's serum it is in association with a pituitary synergist (Evans), which the antiserum inhibits. A similar inhibition of the pituitary synergist of the rat would account for the inhibition of prolan in rats by these antisera.

The injections of the Evans sheep-pituitary preparation (extract No. 2) into a puppy called forth a less effective antiserum. The serum of this animal proved inhibitory only for the extract with which it had been long injected, conforming with the experiences of Collip and his co-workers as quoted in the introductory paragraphs.

The differences in degree of antagonistic power observed in the three sera may possibly be accounted for by the longer period of injections given to the collie dog, or by a natural inability of puppies of this age to produce strong immune bodies or anti-hormones.

It was hoped that a parallel study of the precipitin reactions between these various sera and hormones could be reported along with these biological data. Numerous observations of the sort were made by Dr. Monroe Eaton of the Department of Bacteriology. Briefly, he found that precipitin tests with the materials did not lead to the concise results that were obtained with the gonadotropic tests in rats. Our most potent antisera gave weak precipitin reactions with active extracts, while some normal sera gave equally good precipitin-like reactions. The most effective antiserum had a high cholesterol content, which obscured the readings, even after it had been centrifuged for long periods. The hydrogen ion concentration of the extracts was carefully adjusted, and

controls of the two preservatives, cresol and merthiolate, were made in order to exclude these factors as the cause of the variations. Because of the occurrence of the precipitin-like reactions of normal sera with hormone preparations, Dr. Eaton feels that further observations are needed before dependable interpretation of the experimental data can be made.

The antagonistic power acquired by the sera of the injected animals might be variously interpreted. It might be due (1) to antibodies in the immunological sense of the word, or (2) to a real antihormone present in the original extract or aroused in the injected animal. In either instance, from these studies, the antagonistic factor appears to be specific for gonadotropic hormones of similar action, irrespective of species of origin or method of preparation. Whether or not the antagonistic principle will prove to be antibody or antihormone, its special affinity for gonadotropic substances of all species tested would seem to indicate that the gonadotropic molecules of each source either are identical or have like linkages with the antibody or antihormone-complex.

SUMMARY

Canine sera after prolonged injections of gonadotropic extracts were tested for their ability to inhibit in rats these hormones prepared from several species of animals, including man. The results and interpretation are as follows:—

(1) Gonadotropic extracts prepared by different methods produced different degrees of antagonism in the canine sera after prolonged injections.

(2) A gonadotropic extract of sheep-pituitary glands, after seventeen months of injections into a female collie dog, produced an antagonistic principle in its serum which completely prevented the action in rats of gonadotropic hormones from sheep and hog-pituitary glands, from human urine of the menopause, human urine of pregnancy, and from the serum of pregnant mares.

(3) The normal development of the reproductive system of young rats was held in abeyance so long as injections of the same serum were given.

(4) After four months of injections of a gonadotropic extract of the serum of pregnant mares, a female fox-terrier puppy's serum contained an antagonistic principle which partially checked the action of corresponding amounts of the gonadotropic hormones from sheep and hog-

pituitary glands, from human urine of pregnancy, and from the serum of pregnant mares.

(5) After four months of injections of a gonadotropic extract of sheep-pituitary glands (extract No. 2), another female fox-terrier puppy's serum contained an antagonistic principle which checked the action of the extract with which this puppy was injected (extract No. 2), but did not check the effects of the other gonadotropic preparations.

These phenomena may be ascribed either to: (1) the development in the animals of an antibody in the immunological sense of the word; (2) the presence in the extract injected of an antihormone which was retained in the injected dog; or (3) to the development of an antihormone in the animal.

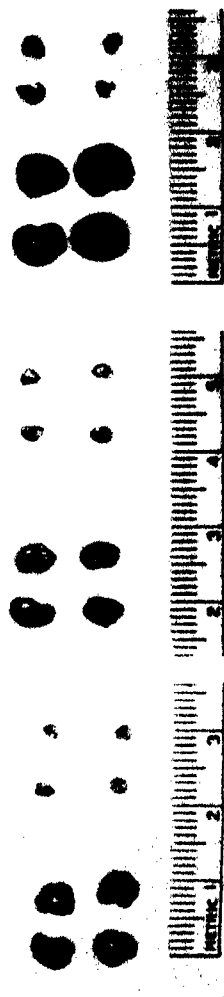
The antagonistic principle in the serum of two animals proved not to be species specific; in other words, these sera checked the action of gonadotropic hormones of several species of animals, including man.

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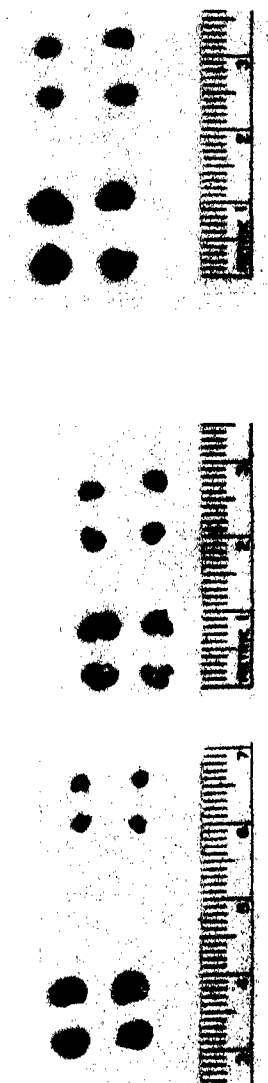
DESCRIPTION OF PLATE 16

FIG. 3—Photographs of ovaries from tests listed in Table I. The pairs of large ovaries on the left in each group are from rats which received a gonadotropic extract and no serum; those on the right are from rats which received the same



a

b



d

e

FIG. 3—*a* to *e*.

FIG. 4.

dose of the same extract plus serum of the collie dog. The extract used in each group was as follows:

- (a) Sheep-pituitary (Collip, Extract No. 3).
- (b) Hog-pituitary (No. 351129BD1).
- (c) Mare pregnancy-serum.
- (d) Human urine of the menopause.
- (e) Human urine of pregnancy (Antuitrin S).

FIG. 4.—Photographs of ovaries of eighty-one-day-old litter-mate rats. The ovaries on the left are from the uninjected controls; those on the right are from rats that received injections of the collie serum for fifty-six days starting at the age of twenty-five days.

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The Effect of Anti-Gonadotropic Serum on the Reproductive Organs of the Normal Animal

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[PLATE 17]

I—INTRODUCTION

It is now well established that anti-gonadotropic activity can be evoked in blood serum by the prolonged injection of animals with gonadotropic extracts, and such extracts from a variety of sources all appear to be effective. Thus extracts of human urine of pregnancy and pituitary extracts were used by Bachman, Collip, and Selye (1934); pituitary extracts by Fluhmann (1935, *a*, *b*), and Eichbaum and Kindermann (1935); pregnant mare serum by Gustus, Meyer, and Dingle (1935), and urine from teratoma testis cases by Twombly and Ferguson (1934).

The recent work of Twombly (1936) lends support to the view that the development of the anti-substances in sera in response to continued administration of hormone preparations is due to the introduction of heterologous proteins with antigenic properties. He has shown that when homologous extracts are injected, such as urine of pregnancy extracts to women, no anti-substances can be detected in the serum. Another relevant experiment was carried out by Du Shane, Levine,

Pfeiffer, and Witschi (1935) who united parabiotically two female rats, one hypophysectomized, the other ovariectomized. The gonadotropic hormone of the pituitary of the ovariectomized one sufficed to maintain the ovaries of the hypophysectomized one in a functional state for over a year without any sign of interference due to the production of anti-hormones. A similar view, based on a comparable experiment, has been put forward by Martins (1935). On the other hand, Gustus, Meyer, and Dingle (1935) prepared from serum of pregnant mares a protein-free extract of gonadotropic hormone which produced an anti-substance in the blood of monkeys, although the latter gave no precipitin reaction with the purified hormone.

The view that the evocation of "antihormones" is due to the administration of heterologous protein matter is incompatible with the view that they occur naturally and play some part in maintaining the endocrine balance in the normal animal. Direct evidence of the natural occurrence of anti-gonadotropic activity is difficult to obtain, but Laroche and Simmonet (1936) have recently reported that the serum of an ovariectomized woman and also that of a patient suffering from oligomenorrhoea showed anti-gonadotropic activity when tested against urine of pregnancy extract on the accessory reproductive organs.

Whether or not anti-bodies to hormones play any part in the normal animal, their practical use will presumably depend on their power to inhibit an animal's own pituitary secretions, and on a lack of species specificity. It has been previously shown by us (Parkes and Rowlands, 1936) that anti-gonadotropic serum, prepared by prolonged injection of gonadotropic extracts of ox anterior pituitary into rabbits, prevents the ovulation-producing activity of similar extracts given to oestrous rabbits. Moreover, ovulation can be prevented by injecting this antiserum after mating, thus showing that the substance inhibits the secretion of the animal's own pituitary. These results appeared to us to warrant further investigation of the effects of the antiserum on the reproductive organs of the normal animal, an aspect of the subject which, especially as regards the male, has been little studied. In several experiments atrophic changes were produced entirely similar to those following hypophysectomy.

II—METHODS

The method of the production of the anti-gonadotropic serum in rabbits has been previously described (Parkes and Rowlands, 1936). Antiserum from the same batch of rabbits was used throughout these experiments. By the end of the time the rabbits had been receiving

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gonadotropic extract daily for about eight months, and the serum was highly anti-gonadotropic.

Details of time and duration of injections, all of which were subcutaneous, are given in their relevant places.

The dissection of the reproductive organs of the male rat was carried out as described by Callow and Deanesly (1935), the organs being weighed on a torsion balance from 70% alcohol after fixation in Bouin's fluid. Material for histological examination was sectioned at 10 μ and stained in Meyer's haemalum and eosin.

III—THE ASSAY OF ANTI-GONADOTROPIC SERUM ON THE IMMATURE RAT OVARY

This serum had previously been used on oestrous rabbits, but it seemed desirable to test it against pituitary extract on immature rats.

Groups of five rats weighing 40–50 gm. were injected for 5 days with varying amounts of anti-gonadotropic serum together with 2 mg. of the extract of horse pituitary (AP2B) examined by Deanesly (1936) (Table I).

TABLE I—THE EFFECT OF VARYING AMOUNTS OF ANTI-GONADOTROPIC SERUM PLUS A CONSTANT AMOUNT (2 MG. DAILY) OF HORSE PITUITARY EXTRACT ON THE IMMATURE FEMALE RAT

Amount of antiserum daily (cc.)	Body weight (gm.)		Weight of organ (mg.)		Number ovulated
	Before	After	Ovaries	Uterus	
0	44	53	59	55	5
0.05	42	48	32	112	2
0.1	44	56	21	92	2*
0.2	47	60	21	47	2
0.3	42	53	19	95	2
0.35	44	56	21	125	2
0.5	47	62	10	—	0
1.0	39	49	11	—	0
Normal rabbit serum	45	52	68	68	5
0.5 cc.					

* Only 4 rats in this group.

It can be seen that the injection of 0.05 cc. of the anti-gonadotropic serum daily halved the response to a total of 10 mg. of the extract, while 0.5 cc. of serum daily abolished the weight response (*see* fig. 1). 0.05 cc. serum daily prevented ovulation in two of the five rats, but 10 times this quantity of antiserum was required to inhibit ovulation in a whole

group. This inhibition is not produced by large amounts of normal rabbit serum.

Microscopic examination showed that even when large amounts of serum were given (0.5 cc. daily) with the 2 mg. pituitary extract, the ovaries contained numerous large follicles measuring about 0.5 mm. in diameter, which is well above the resting size in the rat. No corpora lutea were present in these ovaries. Another group of five rats was given 1 cc. serum and the standard amount of pituitary extract daily for 8 days, and, although large follicles were present, ovulation still did not occur.

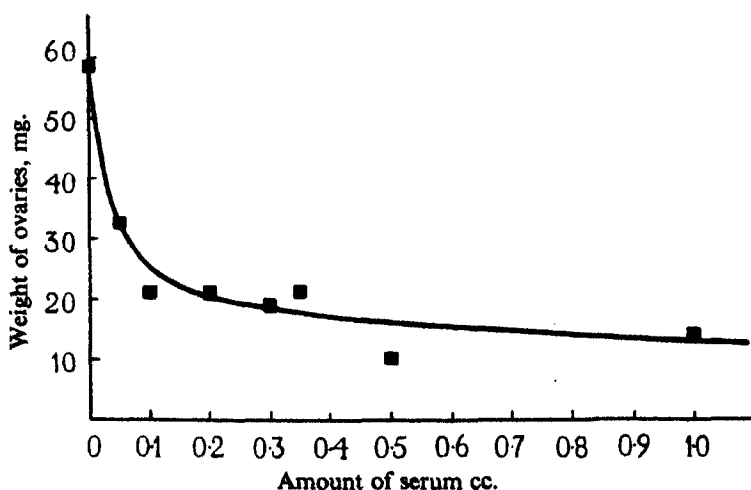


FIG. 1—The effect of varying amounts of anti-gonadotropic serum and a constant amount of horse pituitary extract on the weight of the ovaries of the immature rat.

This particular anti-gonadotropic serum has previously been shown to be much less effective in inhibiting the power of a urine of pregnancy extract to cause ovulation in the rabbit (Parkes and Rowlands, 1936), and it seemed desirable to re-examine this partial specificity using a different test object. A standard amount (0.2 mg.) of urine of pregnancy extract (UP 10) was injected daily for 5 days with different quantities of the anti-gonadotropic serum (Table II and fig. 2). According to Deanesly (1936), this amount of extract produces ovaries weighing about 40 mg.

It is clear that the anti-gonadotropic serum considerably inhibited the activity of urine extract; 0.5 cc. of the anti-serum daily prevents most of the increase in weight of the ovary and inhibited ovulation in four of the five animals.

In both sets of experiments there is some suggestion that, where larger

doses of serum are given and the gonadotropic extracts are more completely inhibited, the uteri of the test animals are larger. This might be due to the serum being faintly oestrogenic, though no evidence of this has been obtained; it is more likely caused by the selective neutralization by the serum of the luteinizing activity of the extracts.

TABLE II—THE EFFECT OF ANTI-GONADOTROPIC SERUM ON THE RESPONSE OF THE IMMATURE RAT OVARY TO 1 MG. OF URINE OF PREGNANCY EXTRACT (UP 10)

Amount of antiserum given daily (cc.)	Body weight (gm.)		Weight of organ (mg.)		Number ovulated
	Before	After	Ovaries	Uterus	
0	42	52	34	82	5
0.1	44	63	29	85	4
0.25	45	56	22	137	4
0.5	45	57	17	127	1
1 cc.	47	60	42	103	5
normal rabbit serum					

5 rats in each group.

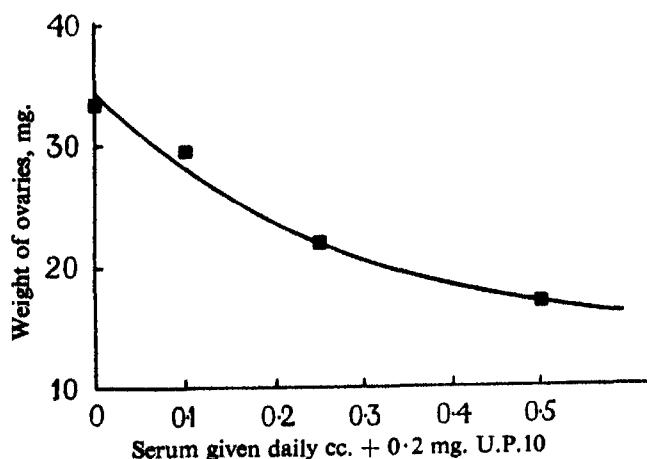


FIG. 2—The effect of varying amounts of anti-gonadotropic serum and a constant amount of urine of pregnancy extract on the weight of the ovaries of the immature rat.

IV—EFFECT ON THE DONOR

In considering the action of anti-gonadotropic serum on the effectiveness of the animal's own pituitary, attention should first be directed to the

donor animal. It has been shown that 5 cc. of the serum will inhibit ovulation in eight out of ten mated oestrous rabbits. The donor rabbits possess about 100 cc. of this serum each—what is the effect on their own reproductive organs? The first donor rabbits, killed after about 5 months of injection, had fairly normal oestrous ovaries and their uteri were not atrophic. The antigenic extract, however, was made from ox pituitary, which, from the work of Hill (1934), is known to contain relatively large amounts of luteinizing hormone and little follicle-stimulating hormone. Hence it was reasonable to suppose that the antiserum contained mainly anti-luteinizing substance, and that this had not affected follicular growth. Such immunized rabbits with large follicles should therefore mate but not ovulate.

To test this tentative conclusion, mating experiments were carried out on another set of rabbits injected for about four months with cow pituitary extract. Six of these mated readily, of which only one ovulated. The serum of this rabbit was subsequently tested separately and was found to have less anti-gonadotropic power than the usual mixed batches of serum.

A further batch of donor rabbits killed after eight months of injections contained many animals whose reproductive organs were atrophic, resembling those of hypophysectomized animals. It is probable that by this time effective amounts of antibody to follicle-stimulating hormone had also developed.

These experiments, which receive support from further results described in this paper, imply that there is an immunological difference between the follicle-stimulating and the ovulation-producing or luteinizing substances and provide added evidence of their separate identities.

V—EFFECT OF ANTI-GONADOTROPIC SERUM ON THE REPRODUCTIVE ORGANS OF THE ADULT MALE RAT

(a) *The Effect on the Normal Rat*—It is now well known that extracts of the pituitary body cause activity and hypertrophy of the interstitial cells of the testis, and that stimulation of the accessory reproductive organs follows from the increased secretion of male hormone. According to Smith, Engle, and Tyndale (1934), and Greep, Fevold, and Hisaw (1936), this response is brought about by the luteinizing substance of the pituitary, and Kuschinsky and Tang-su (1936) remark on the parallel effects of the luteinizing substance of pregnancy urine on the male and female interstitial tissue. Since the anti-gonadotropic serum used in the present experiments seemed to contain predominantly the anti-

luteinizing substance, the effect of this serum on the adult male reproductive tract was investigated. Groups of five adult male rats about 140 gm. weight were injected twice daily for 14 days with varying amounts of serum. The rats were killed 24 hours after the last injection.

The results are given in Table III and in fig. 3, from which it can be seen that the regression of the seminal vesicles and prostate is greater than that of the testes. The atrophy of the accessory organs is very rapid when 2 cc. of the antiserum is given daily. When 3 cc. daily is given there is little additional loss in weight in the accessory organs, but the testes decrease further in weight. Presumably, high doses of the antiserum are required to affect the spermatid tubules, which, of course, make up the main bulk of the testis. Histological examination showed that the larger amounts of the antiserum caused aspermia, but numerous spermatids persisted in the tubules, fig. 5, Plate 17.

Since it appears very probable that the interstitial cells of the testes secrete the male sex hormone which maintains the accessory reproductive organs in a functional state, the effect of simultaneous administration of male hormone and anti-gonadotropic serum was tried. Five adult male rats were injected with 0.75 cc. of antiserum twice daily together with 300 γ of androstanediol in oil once daily for 14 days. From the data of Deanesly and Parkes (1936) and Callow and Deanesly (1935), this amount of androstanediol should maintain the seminal vesicles and prostate of the post-pubertally castrated rat at normal size. The mean weights of the organs of these rats are given in Table III. The testes were found to be below normal weight, but the accessory reproductive organs were completely maintained.

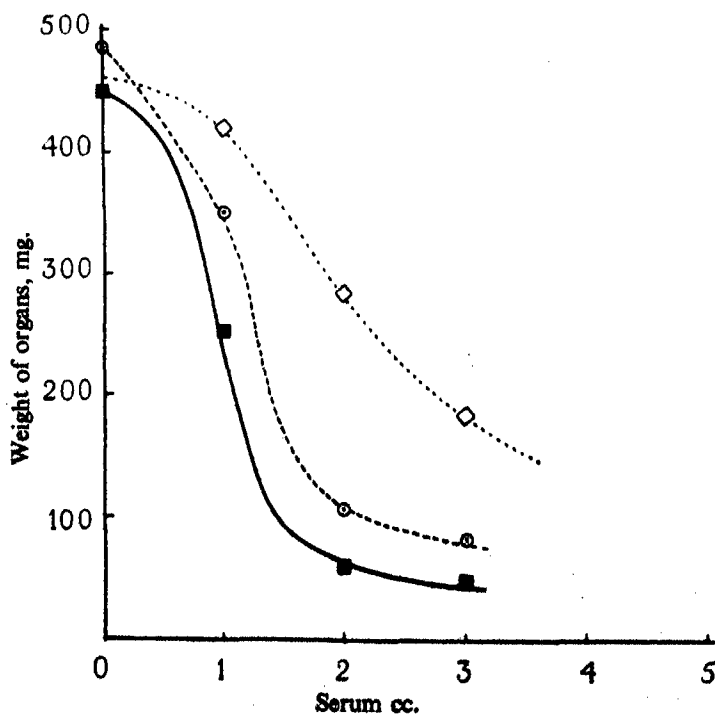
(b) Comparison of the Rate of Regression of the Reproductive Organs of the Male after Various Treatments—The atrophic changes in the reproductive organs were so rapid after administration of anti-gonadotropic serum that a comparison was made with the rate of atrophy after castration and after hypophysectomy. The duration of administration of serum and the time after castration and hypophysectomy was 14 days in all animals.

Table III shows that the rate of atrophy of the accessory organs caused by 3 cc. daily of the anti-gonadotropic serum is similar to that caused by hypophysectomy and castration. The effect of the antiserum on the weight of the testes is not so great as that of hypophysectomy. This differential effect is easily explained if one accepts (a) the supposition that antiserum to ox pituitary is mainly anti-luteinizing hormone, and (b) the view that the endocrine activity of the testes is controlled by the

TABLE III—THE EFFECT OF VARIOUS TREATMENTS ON THE REPRODUCTIVE ORGANS OF THE ADULT MALE RAT. IN EACH CASE THE TREATMENT LASTED 14 DAYS

Treatment	Body weight (gm.)			Weight of organs	
	Before	After	Testes (gm.)	Seminal vesicles (mg.)	Prostate (mg.)
None	—	162	—	474	511
3 cc. normal rabbit serum daily	151	162	2.26	341	419
1 cc. anti-gonadotropic serum daily	140	164	2.10	252	349
2 cc. anti-gonadotropic serum daily	156	177	1.42	59	107
3 cc. anti-gonadotropic serum daily	134	151	0.91	48	87
1½ cc. anti-gonadotropic serum daily and 300 γ androstenediol	144	171	1.72	500	510
Castration	148	169	—	69	106
Hypophysectomy	142	108	0.65	51	82

5 rats in each group.

FIG. 3—The effect of anti-gonadotropic serum on the reproductive organs of the adult male rat. --- testes $\times \frac{1}{2}$; — seminal vesicles; — — — prostate.

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luteinizing hormone and the spermatic tubules by the follicle-stimulating hormone.

VI—THE EFFECT OF ANTI-GONADOTROPIC SERUM ON THE REPRODUCTIVE ORGANS OF THE NON-PREGNANT FEMALE

(a) *Inhibition of Ovulation in the Pubertal Rat*—Five female rats, weighing 60–70 gm., in which the vagina was still closed, were injected twice daily with 1 cc. of anti-gonadotropic serum. After 10 days the mean body weight in the group had increased from 66 to 79 gm. and the serum thus showed no signs of an anti-growth activity. The vagina of each was showing signs of opening, but in none was it fully patent. The vagina opens at 80–90 gm. body weight in this strain of rats (Deanesly, 1936), so that the partial opening in these rats was normal. At this time the right ovary of each rat was removed; the average ovarian weight was 7 mg., which is not appreciably lower than normal.

Since ovulation in the normal rat takes place shortly after the opening of the vagina, it was decided to continue the injections until the body weight had increased to about 120 gm., at which weight normal rats have ovulated. The duration of the period of injection was therefore varied as shown in Table IV.

TABLE IV—EFFECT OF ANTI-GONADOTROPIC SERUM ON THE PUBERTAL RAT

No. of rat SH	Period of injection (days)	Body weight (gm.)		Weight of ovaries (mg.)		Weight of uterus (mg.)	Mean diameter of 6 largest follicles (mm.)
		Before	After	Right	Left		
64	39	70	125	7	14	82	0.61
65	61	66	117	7	13	50	0.58
66	45	63	121	8	16	53	0.74
67	45	68	122	7	14	150	0.75
68*	10	74	83	14		68	—

* Died under anaesthetic at operation after 10 injections.

The remaining left ovary of each animal had doubled its weight since the time of the removal of the right ovary, and had therefore maintained a practically normal weight (Deanesly, 1936). The vaginae were all open.

Macroscopically it was seen that, though none of the rats had ovulated, the ovaries were full of large follicles, fig. 7, Plate 17, and the uterus of

SH 67 was in an oestrous condition. The absence of ovulation was confirmed by histological examination. Large follicles were present in all ovaries, the mean diameter of the six largest being given in Table IV. The mean diameter of the follicle in the rat at the time of ovulation is about 0.9 mm. (Parkes, 1931), one or two individual follicles in SH 66 and 67 were found to be approaching this size, but the largest in SH 64 was only 0.65 mm. The vagina of SH 64 showed an epithelium of several layers of stratified cells and there were frequent areas where the cells were highly mucified. The vagina of SH 66 also showed mucification but to a lesser degree, while that of SH 67, whose uterus showed the typical oestrous distention, was cornified.

Five more rats of similar body weight were injected daily with the same amount of normal rabbit serum. After 16 days the average body weight was 99 gm. with a range of 91–111 gm.; the vagina was fully open in each. On killing, the ovaries, which had an average weight of 33 mg., all contained numerous large corpora lutea, fig. 6, Plate 17. Comparison of these two groups, therefore, leaves no doubt that ovulation had been indefinitely delayed in those receiving anti-gonadotropic serum.

(b) *The Effect on the Adult Rat*—It was hoped that, since the anti-serum contained mainly anti-ovulating substance, continued injection of this substance would suppress ovulation and the formation of corpora lutea and would allow the animal to assume a condition of constant oestrus.

Three adult female rats of mean body weight 158 gm. were injected with 1 cc. of anti-gonadotropic serum twice daily for four weeks. During this time the average body weight increase was 4 gm. The mean weight of ovaries was 41 mg. and on section they were found to contain normal follicles and corpora lutea of uncertain age. Ovulation may or may not have been suppressed, but although cornification of the vagina persisted in one rat for 4–5 days there was certainly no evidence of a state of persistent oestrus. These rats had normal uteri.

(c) *The Effect on the Adult Mouse*—The experiment was repeated on groups of five mice of about 25 gm. body weight, which were injected with varying amounts of anti-gonadotropic serum (Table V).

Ten days after the first injection the right ovary of each animal was removed, but histological examination of these showed little or no effect of treatment. Injections were continued for another 10 days before the animals were killed. Table V gives the weight of the uterus together with measurements of the largest follicle and corpus luteum in the ovary at autopsy. The mean is obtained by measuring the two largest dia-

meters at right angles to each other. It can be seen that there is a fall in the uterus weight; 0.2 cc. daily causes a slight drop, while 0.5 cc., given either as a single daily injection or divided into two and given at 12-hour intervals, has a marked effect.

Although it is only possible to detect a very small difference in the size of follicle as the result of injection of anti-gonadotropic serum, it would appear to be significant because of the atrophic changes in the uterus. Compared with the results of Brambell and Parkes (1927), the diameters of the follicles are, however, less than at ovulation (0.55 mm.).

TABLE V—EFFECT OF ANTI-GONADOTROPIC SERUM ON THE ADULT FEMALE MOUSE

No. of mice	Amount of serum given daily (cc.)	No. of daily injections	Weight of uterus (mg.)	Diameter of largest	
				Follicle (mm.)	Corpus luteum (mm.)
SH 34-38	0.2	2	67	0.44	0.59
SH 39-43	0.5	2	28	0.38	0.54
SH 44-48	0.5	1	31	0.37	0.53
SH 49-53	0.5	2	82	0.49	0.60
normal serum					

The condition of the corpora lutea present was compatible with there having been no ovulation during the later part of the injection period, but it is difficult to say whether ovulation had been entirely suppressed. Under certain conditions, old corpora lutea may persist almost indefinitely in the mouse ovary.

VII—THE EFFECT OF ANTI-GONADOTROPIC SERUM DURING PREGNANCY

(a) *The Rabbit*—Experiments were carried out on rabbits at various stages of pregnancy, since it is known that in this species the pituitary is essential for the maintenance of the foetuses throughout gestation (Smith and White, 1931; White, 1933; Firor, 1933; Robson, 1935). A group of 12 rabbits of the same breed were mated, ovulation being confirmed by a sterile laparotomy 24 hours later, and starting 48 hours after mating they were injected subcutaneously for 8 days with the anti-gonadotropic serum and killed the following day (Table VI).

It will be seen that the number of animals in which foetuses failed to become implanted increased with the quantity of antiserum injected.

AGR 24, which failed to become pregnant when given 2 cc. of anti-serum, had one large corpus luteum, only, but there was considerable proliferation of the endometrium. The other rabbits non-pregnant at autopsy had no corpora lutea in the ovaries and there was no progestational uterine proliferation.

TABLE VI—EFFECT OF ANTI-GONADOTROPIC SERUM ON IMPLANTATION
IN THE RABBIT

No. of rabbit AGR	Amount of anti-gonadotropic serum given daily (cc.)	No. of foetuses implanted
21	0	4 + 2
22	0	3 + 1
24	2	None
25	2	3 + 4
26	2	6 + 3
27	5	3 + 3
28	5	None
29	5	None
41	10	None
42	10	None
43	10	None
23	5 cc. normal rabbit serum	3 + 1

Since laparotomy 24 hours after mating might possibly have interfered with the passage of the ova through the Fallopian tubes, it was decided to repeat this experiment, performing the operation 48 hours after mating, when the ova are at the lower end of the tube and safe from any injury.

Four rabbits were mated, laparotomized 48 hours later, and then injected immediately with 2.5 or 5 cc. of the anti-gonadotropic serum twice daily for 8 days. They were killed 24 hours after the last injection. In those injected with a total of 5 cc. daily there was no sign of implantation having taken place, the ovaries were devoid of corpora lutea and the uterus had not proliferated. The mammary gland of one of these consisted of areas about 2½ inches in diameter, with an actively growing ring of tissue at the periphery. The mammary gland of the other rabbit was full of milk, indicating a breakdown from a previous pseudo-pregnancy.

Of the two rabbits injected with 5 cc. of the antiserum twice daily, one had implanted. There were three foetuses in the left horn and five in the right, the corpora lutea appeared normal and the endometrium was highly proliferated. The second rabbit failed completely to become pregnant.

A further set of experiments was carried out on rabbits at a later stage of pregnancy (Table VII). The daily dose of antiserum was given as two injections, twelve hours apart. Injections were continued for 7 days and the animals killed 24 hours later.

TABLE VII—THE EFFECT OF THE ANTI-GONADOTROPIC SERUM ON THE YOUNG FOETUS OF THE RABBIT

No. of rabbit AGR	Time of 1st injection post-coitum (days)	Total amount of anti-gonadotropic serum given daily for 7 days (cc.)	Result
52	13	2	Normal
53	13	2	Normal
54	15	2	Normal
59	13	5	Complete resorption
55	15	5	Complete resorption
56	13	5	Incomplete resorption 3 + 0 normal, 2 — 1 resorbed
57	15	5 cc. normal rabbit serum	Normal
58	15	"	Normal
60	15	"	3 + 4 resorbing otherwise normal
61	15	"	1 resorbing otherwise normal
62	15	"	Normal

It can be seen from Table VII that a total of 2 cc. serum daily failed to disturb pregnancy, but the injection of 5 cc. daily caused complete resorption of foetuses in two of three rabbits. Occasional resorption is common in rabbits when the number of foetuses is large, as in those injected with normal rabbit serum, but complete resorption under normal conditions is rare. It seems highly probable, therefore, that the anti-gonadotropic serum interfered with pregnancy in two cases by causing resorption of the foetuses.

Histological examination of the ovaries and uterus of AGR 55 and 59 was carried out. Sections were cut at the site of implantation, characterized by the presence of a band of hypertrophied muscle about 1 cm. in width encircling the placental site. No trace of foetal remains could be found in the uterus; the endometrium in this region was very oedematous and contained large numbers of placental giant cells which are common in the rabbit during this stage of pregnancy (Sansom, 1927). The ovaries contained small degenerate corpora lutea and a few small follicles.

(b) *The Rat*—The results obtained with anti-gonadotropic serum on the pregnant rabbit resemble those following hypophysectomy. The effect

on the pregnant rat was then studied, because in this species the normal development of the foetuses is maintained after hypophysectomy in the second half of pregnancy, and, although gestation may be prolonged in some, parturition is normal (Selye, Collip, and Thomson, 1933, *a*; Pencharz and Long, 1933).

Five rats were injected subcutaneously once daily with 2 cc. of anti-gonadotropic serum from the 13th day post-coitum onwards. Two were killed on the 21st day post-coitum, and in these the foetuses were all living and appeared normal. The ovaries contained large corpora lutea while the mammary glands were well developed. The other three were injected until parturition; two gave birth to normal litters on the 21st day post-coitum, and the remaining one on the 23rd day post-coitum. The last litter and one of the young born 21 days post-coitum were destroyed by the parent on the following day. Another 5 rats were injected, commencing at the same time, with 1.5 cc. of serum at a 12-hour interval until parturition. This again failed to prevent the birth of normal litters 21–24 days post-coitum.

It can be safely stated, therefore, that during the second half of pregnancy in the rat, the anti-gonadotropic serum is without effect.

(*c*) *The Mouse*—Selye, Collip, and Thomson (1933, *b*) found that hypophysectomy during the later stages of pregnancy in the mouse does not prevent parturition, but does result in the young being born dead; the regression of the corpora lutea is more rapid than in the rat under similar circumstances.

Four pregnant mice on the 13th day post-coitum were injected subcutaneously once daily with 0.5 cc. anti-gonadotropic serum. Pregnancy was not interfered with, and all mice produced normal, living litters on either the 19th or 20th post-coitum. In the same way 0.5 cc. of anti-gonadotropic serum given twice daily at 12-hour intervals failed to disturb pregnancy in two mice, which produced litters on the 21st day post-coitum. The litters when first found had been partly destroyed by the mothers, so that it is uncertain whether they were born alive.

It appears, therefore, that the effect of the antiserum on the corpus luteum of pregnancy in the mouse has not been sufficient to interfere with the production of living young, and the results are not comparable with those following hypophysectomy.

VIII—THE EFFECT OF ANTI-GONADOTROPIC SERUM DURING LACTATION

(*a*) *The Rat*—The rats that had been injected with anti-gonadotropic serum from the 13th day post-coitum and had produced normal living

young were injected after parturition with 1·5 cc. of the antiserum twice daily for 10 days at 12-hour intervals. All five rats lactated and the young suckled and survived, except four in one litter of seven.

(b) *The Mouse*—Similarly, the pregnant mice after giving birth to normal litters were injected with 0·5 cc. of the anti-gonadotropic serum twice daily. The serum again failed to prevent the mothers lactating and the young suckled and developed normally.

The negative effect on lactation is somewhat surprising, since it is known (unpublished data) that the extract from which the anti-gonadotropic serum was prepared contained considerable quantities of lactogenic hormone. Further work, however, is being carried out to investigate the anti-lactogenic activity of this antiserum, using the pigeon crop-gland as a test-object.

It is with very great pleasure that I acknowledge my best thanks to Dr. A. S. Parkes, F.R.S., for every assistance throughout this work, and in the preparation of the manuscript. I also wish to thank Dr. R. L. Noble of the Middlesex Hospital Medical School for providing me with the hypophysectomized rats used in this work. The androstanediol used was kindly made available by Messrs. Ciba of Basle, while large amounts of desiccated ox pituitary were provided at a special price by the British Drug Houses.

SUMMARY

Anti-gonadotropic rabbit serum, prepared by the prolonged injection of ox anterior pituitary extract, was found to:—

- (a) Inhibit ovulation and corpus luteum formation in the donor rabbits.
- (b) Cause atrophy of the reproductive organs of the adult male rat. The rate of atrophy of the accessory reproductive organs was similar to that following hypophysectomy or castration.
- (c) Suppress ovulation in the pubertal rat.
- (d) Inhibit implantation of the blastocyst in the rabbit or cause resorption of the foetuses if injections were begun later.

All these effects are similar to those observed after hypophysectomy.

The serum also inhibited the action of horse pituitary extract on the immature rat, and to a lesser degree, the action of urine of pregnancy extract.

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The serum failed to:—

- (a) Suppress corpus luteum formation in the adult rat and mouse.
- (b) Interfere with pregnancy and lactation in these two species.

DESCRIPTION OF PLATE 17

FIG. 4—Testis of normal adult rat. $\times 132$.

FIG. 5—Testis of adult rat treated with 3 cc. anti-gonadotropic serum for fourteen days. The spermatid tubules are greatly reduced in diameter and spermatozoa are absent. $\times 130$.

FIG. 6—Ovary of rat, (SH 79; 100 gm. body weight) injected with 2 cc. normal rabbit serum daily for eighteen days. The corpora lutea and follicles are normal. $\times 18$.

FIG. 7—Ovary of rat (SH 67; 122 gm. body weight) injected with 2 cc. anti-gonadotropic serum daily for forty-five days, showing absence of corpora lutea. Ovulation has been inhibited. $\times 18$.

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FIG. 4.



FIG. 5.

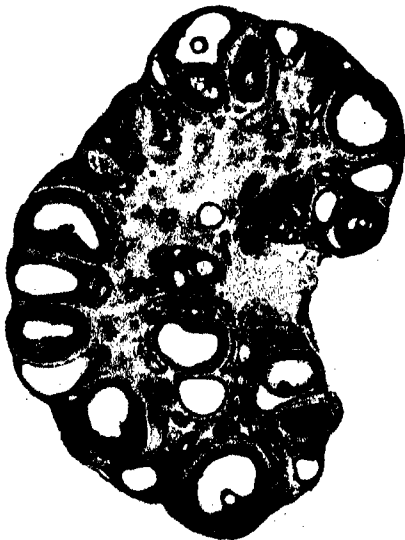


FIG. 6.



FIG. 7.

**An Anomalous Fossil Organism, Possibly a New Type of
Chordate, from the Upper Silurian of Lesmahagow,
Lanarkshire—*Ainiktozoon loganense*, gen. et sp. nov.**

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[PLATES 18–22]

INTRODUCTION

The specimens forming the basis of the present paper were all found in the Ludlow Beds of Logan Water, Lesmahagow, Lanarkshire. The place where they were collected is about half a mile above the Logan Reservoir, on the east side of the upper part of Logan Water. It is almost certain that they came from the beds that are described as No. 3, *Ceratiocaris* beds, of the Ludlow series in "The Silurian Rocks of Britain," vol. 1, Scotland.* They were collected by the members of a working geological party under the direction of the late Professor Peter Macnair round about the year 1904, some certainly in that year as shown by the date scratched upon them. Possibly one was found as early as 1899, if the paragraph on p. 208 of Macnair's paper, entitled "Camp Siluria" (Macnair, 1905), commencing "Another interesting specimen . . .", may be regarded as referring to the organism now to be described. Most of the specimens were collected by D. Nimmo, but others were also found by J. Young, W. Fletcher, and possibly other members of the party referred to. They all appear to have been handed to Macnair. After the latter's death they passed into the charge of the late Professor J. W. Gregory, by whom they were sent to me for examination and report. They belong to the Hunterian Museum, University of Glasgow.†

Before the specimens came to me, the only published reference to them, except for the possible reference mentioned above, was a few words at the beginning of a paper on *Cyamocephalus* by the late L. D. Currie (1927). In December, 1929, some of them were, with Professor Gregory's approval, exhibited by me before the Geological Society of London (Scourfield, 1929–30). For some months in 1931–32, with the

* 'Memoirs of the Geological Survey of the United Kingdom' (1899).

† Certain specimens have now been presented by the Hunterian Museum to the British Museum (Natural History). See p. 545.

consent of Professor E. B. Bailey, who had succeeded Professor Gregory in the Chair of Geology at Glasgow University, some specimens were exhibited in the Geological Department of the Natural History Museum, South Kensington, as part of a special exhibit of "Problematica".

The total number of specimens dealt with is 29, of which five are counterparts of five of the others. It seems that the collection originally consisted of 32 specimens, numbered 1 to 32, but two (Nos. 12 and 22)* were not included among those sent to me and cannot be traced, while one (No. 31) does not belong to the organism to be described. It would appear probable that the specimens, recognized as being peculiar, have always been kept together and none distributed. So far as can be ascertained, there are no other specimens of the same organism in any museum or private collection. Naturally the specimens are in very different states of preservation, many very poor and fragmentary, but the better ones relatively well preserved and showing a considerable amount of detailed structure. The matrix in which they are embedded is a rather fine-grained shale similar to that containing specimens of *Ceratiocaris*, *Pterygotus*, etc., from the same locality, to be seen in many geological collections.

DESCRIPTION OF THE SPECIMENS

A little careful examination shows that all the specimens belong to the same type of organism, possibly even to the same species, and a comparison of the accompanying photographs (Plates 18-22) will, I believe, make this sufficiently clear, at any rate in the case of the specimens illustrated. Taken together they seem to represent a fairly complete organism and not to be merely part of some larger animal. The considerable range in size does not appear to be associated with any evident difference in structure. One point which is very noticeable is that all the specimens are fossilized in exactly the same position, presenting, apparently, a side view and showing that the organism, regarded as a whole, was comparatively thin in relation to its length. In their present condition they are very thin indeed and scarcely penetrate to any appreciable depth into the matrix.

As names of some sort must be used in the detailed description which follows, it has been thought advisable to use non-committal terms for the various parts, such as "capsule", "segmented rod", "fascicle", etc., their true nature being still a matter of some uncertainty.

* The specimen numbers quoted throughout this paper are the original temporary numbers. The corresponding official registration numbers are shown on p. 545.

The "*Capsule*"—The structural feature which is most constant and most striking is a more or less ovoid body truncated at one end, referred to in this paper as the upper end, and rounded at the other. It is present in no fewer than 25 out of the 29 specimens, the remaining four being portions of other parts of the organism. This body, which it is proposed to call the "*capsule*", ranges in size in the different specimens from just under one inch to nearly three inches. (See photographs 1-13, Plates 18-21, * all natural size and in all of which the capsule is the central feature.) It seems to be composed of an inner and an outer layer often separated by an interval of one-sixteenth of an inch or more. The inner layer, in some cases at least, appears to be constricted at the upper end, while the outer layer runs up into the truncated part, presumably forming its boundary on either side. The truncated upper part of the capsule is composed of a series of nearly straight vertical bars, often broader than the spaces between them, which it is proposed to refer to as the "*palisade*". Projecting obliquely above the palisade in one specimen (No. 32, photograph 1, Plate 18), there are some delicate but sharply defined branching filaments which will be referred to as "*cirri*". Similar filaments also appear to be indicated in one other specimen (No. 5, photograph 5, Plate 20) though not so clearly. The elements of the palisade appear in most specimens to be continued down over the middle and lower surface of the capsule radiating somewhat as they do so and usually losing their bar-like character, becoming slightly undulating and tapering lines. They extend to the outer margin of the bottom of the capsule. In the more or less triangular space between the inner and outer layers, just below the palisade, there appear, in at least two specimens (No. 21, photograph 9, Plate 21, and No. 29), a group of similar lines radiating obliquely from the inner part of the capsule outwards and upwards.

The "*Segmented Rods*"—The next most striking feature, at any rate in the best preserved specimen (No. 32, photograph 1, Plate 18), is the presence on one side of the capsule of two converging segmented tapering rods. The presence of such rods, in a more or less imperfect condition, is evident in some half-dozen specimens (Nos 2, 4, 21, 24, 30, and 32, photographs 8, 12, 9, 14, 13, 1, Plates 21, 22, 18) and no doubt occurred

* The photographs were taken with the specimens immersed in a shallow bath of methylated spirit. By this means the disturbing effects of the irregularities of the surface of the matrix are much reduced and the black material of the fossils stands out more plainly than when photographs are taken directly from the dry specimens. I wish to thank Mr. A. W. Dennis for the great care in the preparation of the photographs.

in all. Wherever they can be traced, they are always inclined to one another at practically the same angle, namely, about 25° .

The lower rod is stouter and more strongly marked than the upper and may be referred to as the "primary segmented rod". It starts abruptly just within the edge and a little below the middle of the capsule from which it projects as a rule almost at a right angle. (Most clearly seen in No. 32, photograph 1, Plate 18.) The segments of which it is composed are approximately equal in length though diminishing in breadth as they recede from the capsule. They are covered with closely set lines or bars, somewhat resembling those of the palisade, running lengthwise. The middle of each segment may show some constriction and is marked by a transverse line or perhaps a slight break in the continuity of the longitudinal bars. On the lower side, at or near the junction of each pair of segments, there arises a strong spine, divided at the base into two branches, thus producing a bifurcated effect (Nos. 19, 21, 24, 27, and 32, photographs 4, 9, 14, 6, 1, Plates 19, 21, 22, 18). The spine nearest the capsule seems to be divided to a much greater extent than the others. Whether these spines would have shown their bifurcated bases in a side view of the living organism is uncertain. They may very well have been rotated through 90° in the flattening process of fossilization. The length of the spines is greatest at the proximal end of the rod, *i.e.*, nearest the capsule, decreasing gradually towards the distal end, but apparently always exceeding the width of the segments of the rod.

The upper rod is not so well defined as the lower and may therefore be referred to as the "secondary segmented rod". (Best seen in No. 32, photograph 1, Plates 18, 21, and fairly well in Nos. 2, 9, 21, and 24, photographs 8, 9, 14, Plates 18, 21, 22.) It starts from or quite close to the upper part of the capsule and runs obliquely downwards to meet the primary rod at its extremity. Its segments appear to correspond in number to those of the primary rod and to be finely striated. They are rather widely separated from one another by a basal continuation of the "lobes" to be referred to presently. Between the two rods the structures are not very clear, but they appear to consist mainly of a series of somewhat rhomboidal connecting pieces running obliquely from the primary to the secondary rod and joining on to the basal continuation of the lobes.

The "*Lobes*"—Another very characteristic feature, best seen in No. 26, photograph 2, Plate 18, but more or less evident in several other specimens (*e.g.*, Nos. 2, 4, 5, 21, 24, 30, 32, photographs 8, 12, 5, 9, 14, 13, 1, Plates 21, 20, 22, 18), is a series of nine or ten striated somewhat oval processes arising from the upper edge of the secondary segmented rod.

These will be referred to as the "lobes". The proximal lobe, *i.e.*, the one nearest the capsule and which is actually quite near to the palisade, appears to have been the largest, the others following closely and slightly diminishing in size towards the distal end of the rod. All the evidence seems to show that there was only a single median row of these lobes and that they were not in bilateral pairs. The striae on their surface have a superficial resemblance to the venation of a fern leaflet, but near the edge facing the capsule there is a small area where the lines cross. The base of each lobe appears to be carried down between the segments of the secondary rod and to join a connecting piece.

There can be little doubt that the segmented rods with the spines below and lobes above and the intermediate connecting pieces formed one single structure.

The "*Oblique Bands*"—On the opposite side of the capsule to that bearing the segmented rods, the most definite feature and one that is shown quite clearly in many specimens (*e.g.*, Nos. 1, 2, 7, 19, 21, 30, and 32, photographs 11, 8, 7, 4, 9, 13, and 1, Plates 21, 20, 18), is a series of two to four more or less parallel lines usually forming two narrow bands at slightly varying distances apart, crossing the capsule obliquely from about the middle of its lateral margin to somewhere near the lower margin on the opposite side. These bands are not, however, confined to the capsule, but extend beyond it in both directions. At the upper end they continue in the same oblique direction for a little way beyond the capsule and then suddenly bend down at an angle, often approaching a right angle, for a distance about equal to their length over the capsule, again turning up a little and ending vaguely. At the lower end they continue for a short distance in the same direction as across the capsule and then end vaguely in the midst of what will be referred to later as "*Fascicle No. 1*".

The exact position of these oblique bands on the capsule varies in different specimens. Sometimes it is nearly on the edge, scarcely crossing the capsule at all, and sometimes well out towards the middle, but usually in an intermediate position. The explanation seems obviously to be that these structures were movable with regard to the capsule. As to their nature they may possibly represent two narrow tubes, one on either side of the capsule. This idea seems to be supported at any rate by the specimen (No. 28) transferred from its matrix to a film of cellulose by Professor J. Walton. In this case there is a single narrow band of what is presumably powdery carbon, which stands up as a slight ridge above the surface of the capsule. As this view can be shown, by comparison with the counterpart (No. 21, photograph 9, Plate 21), to be from the out-

side, it follows that the band or tube or whatever it was probably ran outside the capsule.

"*Fascicle No. 1*"—Surrounding the capsule, though in some cases originating within it, or more probably over it, there are a number of more or less distinct bundles of what may be alluded to as fibres, very similar to the bars or lines over the capsule. One of these "fascicles" starts in or over the lower part of the capsule and projects straight, or obliquely, downwards, absorbing in its course the "oblique bands" as already mentioned. (Best shown in No. 32, photograph 1, Plate 18, also to be seen in Nos. 1, 4, 7, 10, 26, 30, etc., photographs 11, 12, 7, 10, 2, 13, Plates 21, 20, 18.) It proceeds in the direction of a peculiar organ which will now be referred to as the "hook", although a definite connexion with the latter is not to be made out with complete certainty in any specimen.

The "*Hook*"—In at least half a dozen specimens (Nos. 2, 3, 5, 10, 27, and 32, photographs 8, 5, 10, 6, 1, Plates 21, 20, 18) there is to be seen, usually at a considerable distance below the capsule, a small but well-defined slightly curved body which usually points in the opposite direction to the segmented rods. This body, which will be referred to as the "hook", is not only sharply defined in outline, but, under a magnifying lens, shows a series of fine parallel ridges and furrows at about 1/150 inch apart, set obliquely to the long axis. In some cases the hook appears as if it were a double structure, but the exact interpretation of this is not clear. The actual position of this organ varies somewhat, but always seems to be related to that of fascicle No. 1, and therefore to the oblique bands, although the definite connexion cannot be demonstrated.

"*Fascicle No. 2*"—The second fascicle of fibres which may be mentioned starts over the lower part of the capsule on the side opposite to the segmented rods. It is well shown in many specimens (e.g., Nos. 3, 7, 20, 21, 27, 28, 30, and 32, see photographs 7, 9, 6, 13, and 1, Plates 20, 21, 18). It projects outwards and obliquely downwards and comes to an end abruptly against what, in four specimens at least (e.g., Nos. 7, 20, 30, and 32, photographs 7, 13, and 1, Plates 20, 21, 18), seems to be a well-marked line or boundary wall more or less parallel to the edge of the capsule. This fascicle is strongly marked and of considerable width, narrowing somewhat as it proceeds outwards. Its length is about the same as the width of the capsule. The position of origin seems to vary, depending possibly upon the position of the oblique bands.

Between this fascicle and the first there are indications on several specimens (e.g., Nos. 7, 30, and 32, photographs 7, 13, and 1, Plates 20, 21, 18) of various structures, but in no case can they be made out with any certainty. Some seem to show curved boundary lines more or less concentric with the outline of the capsule, possibly continued round to join the primary segmented rod near the first spine (Nos. 30 and 23, photographs 13 and 3, Plates 21, 19).

"Fascicle No. 3"—Commencing just above and almost at right angles to fascicle No. 2 near its outer extremity is to be seen in several specimens (e.g., Nos. 20, 26, 29, 30, and 32, photographs 2, 13, and 1, Plate 18, 21) a bundle of fibres which runs more or less parallel to the edge of the capsule and crosses the oblique bands not far from their vague termination.

In the approximately triangular space enclosed by fascicles 2 and 3 and the extension of the oblique bands outside the capsule, there are curved lines indicating structures of some sort, but they are too indistinct to permit of detailed description.

The *"Vague Area"*—Above the prolongation of the oblique bands, and therefore outside the upper part of the capsule and palisade on the opposite side to the segmented rods, there is a rather large area evidently occupied by a certain amount of structure. From no single specimen, however, nor from a combination of several, can any clear idea be obtained as to its true nature. The specimen which covers this area in the most complete way (No. 23, photograph 3, Plate 19) is, unfortunately, in general, in a very bad state of preservation, but it seems to show a flattened oval area surrounded by a number of fine parallel lines with a row of arched spaces above. Except for the latter feature, this arrangement appears to be confirmed by other specimens (e.g., Nos. 26, 29, 30, and 32, photographs 2, 13, and 1, Plates 18, 21).

The *"Shagreen Patch"*—At the farthest extension of the *"vague area"* in specimen No. 23 (photograph 3, Plate 19), and in that only, there is a small sub-triangular patch of minute papillae arranged in symmetrical rows having the appearance of a little piece of shagreen. The papillae are so small that they are only visible to the naked eye in a very good light. Magnified five diameters, they appear as shown in photograph No. 15 (Plate 22). The outline of the patch is not very well defined and it seems probable that it is only part of a larger structure.

The *"Auriculate Organ"*—In the upper proximal corner of the shagreen patch there is a small oval ring broken on one side, giving it an ear-like appearance, which seems to be a definite structure as it is in relief and

crossed by the rows of papillae forming the shagreen (see photograph 15, Plate 22). A very similar structure in approximately the same position occurs in specimen No. 21 (photograph 9, Plate 21), although in this there is no trace of papillae.

The "*Cuticle*"—The actual substance of which the specimens are composed looks like a black carbonaceous material, and this is confirmed by the appearance of the specimen already referred to as having been transferred to a film of cellulose No. 28. Overlying this substance, however, there is to be seen in many places and in many specimens thin pieces of a brittle light-coloured and slightly transparent material which may be referred to as the "*cuticle*". It occurs, apparently, over any part of the organism, and the probable inference is that originally it may have covered the whole. Little pieces have been detached from the surface and examined under the microscope. They appear to be made up of two or three layers of differing structure. One such layer seems to be characterized by plain striations or parallel elements of some sort, and another by exceedingly fine dots or granules (see fig. 1). The material does not dissolve in ordinary acids.

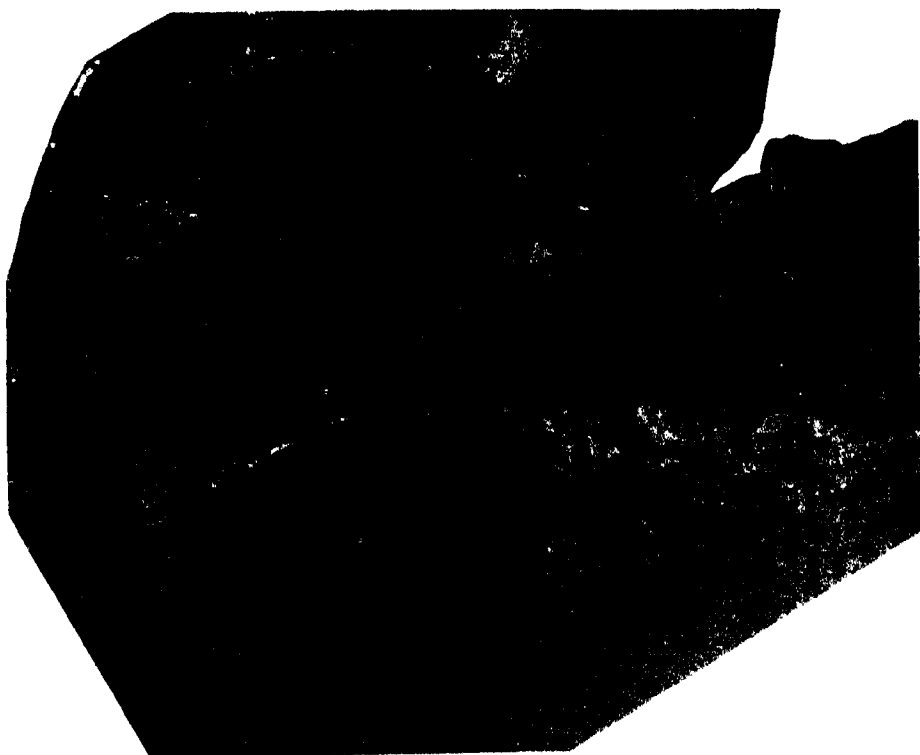
TO WHICH PHYLUM DID THE ORGANISM BELONG ?

In order to facilitate consideration as to its probable nature, an attempt has been made to prepare a diagrammatic reconstruction of the organism represented by the specimens. This is shown in fig. 2. The basis used for the reconstruction was an enlarged photograph of the most complete specimen, viz., No. 32 (shown in photograph 1, Plate 18), and to this was added only such details as could reasonably be justified from the evidence of other specimens, in most cases by the confirmatory evidence of at least two specimens.

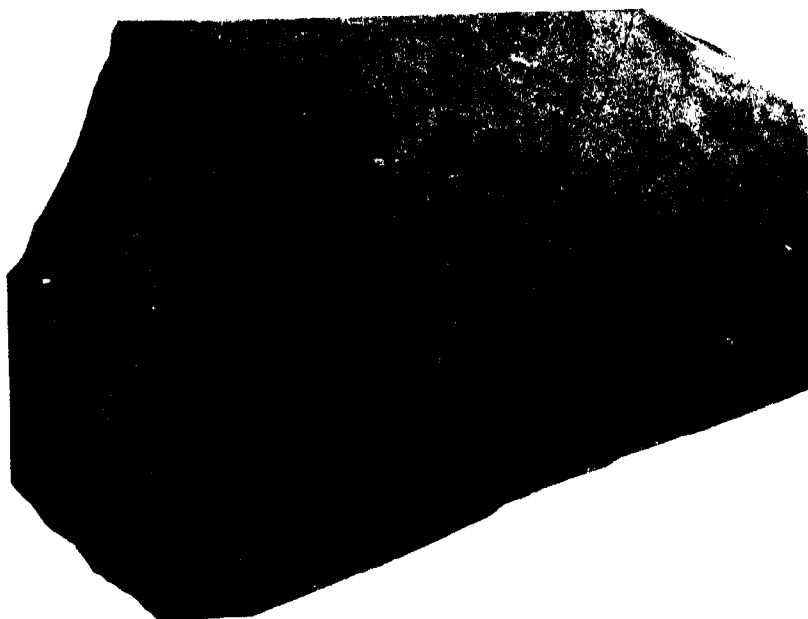
The first question naturally is whether such an organism was plant or animal. In spite of the rather leaf-like appearance of the "*lobes*" and the superficial resemblance of the "*primary segmented rod*" to a stem of *Equisetum*, there seems no reason to suppose that it was a plant. The whole complicated and asymmetrical arrangement of parts speaks against this idea.

But if the organism was an animal, to which of the known phyla did it belong ?

The majority of those who have seen the specimens and who have also been willing to hazard an opinion, seem to think that we have to do with a Crustacean, or at least an Arthropod. It is certainly true that many of



1 (32).

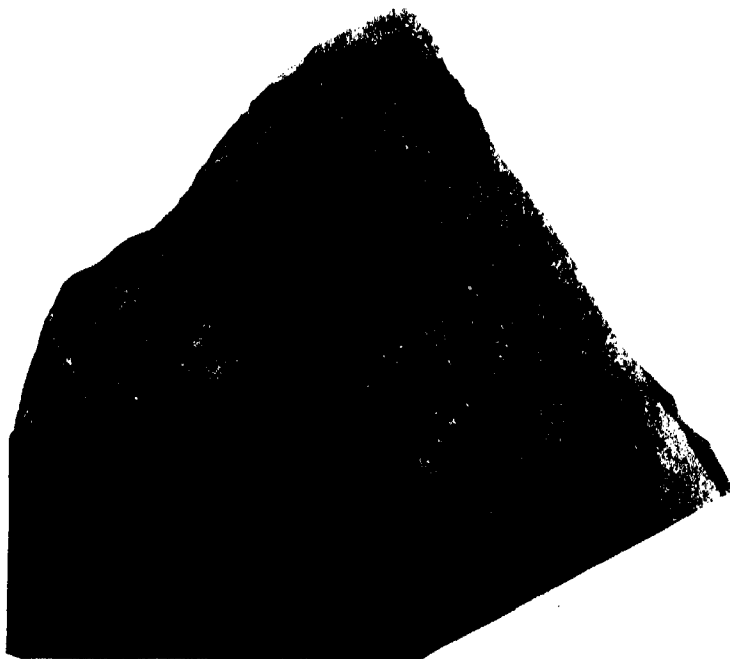


2 (26).

(Facing p. 540)



3 (23).



4 (19).



5 (5).



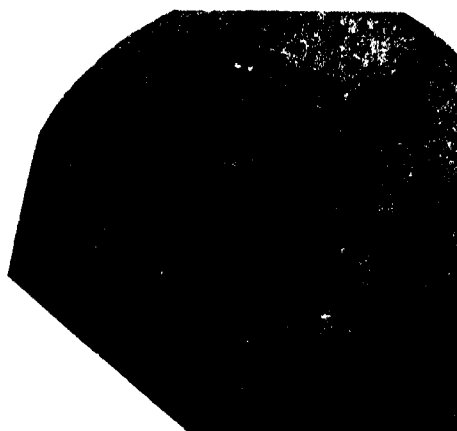
7 (7).



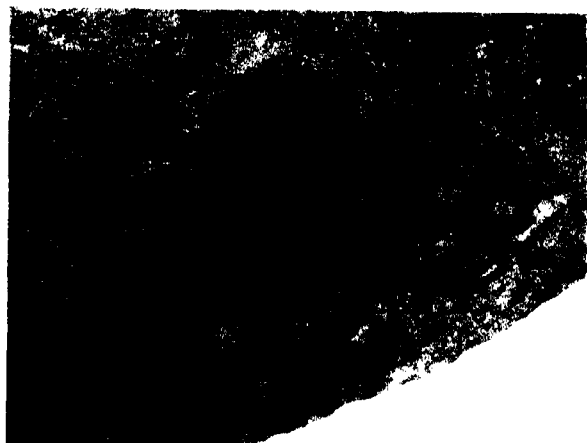
6 (27).



8 (2).



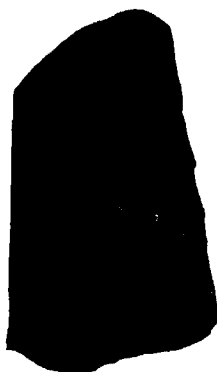
11 (1)



9 (21).



12 (4).



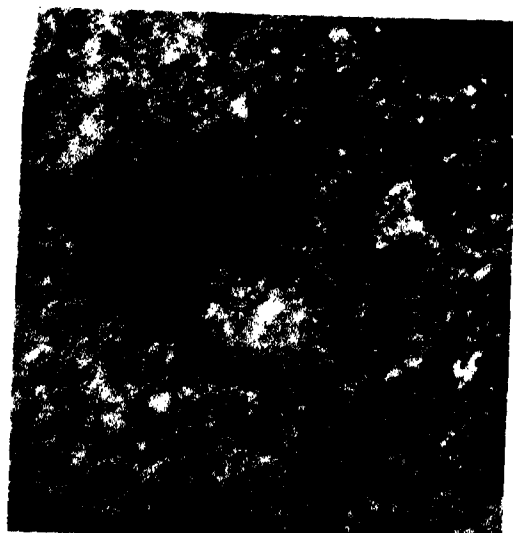
10 (10).



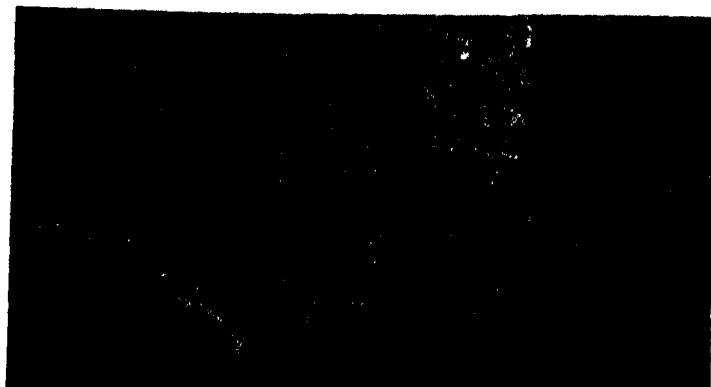
13 (30).



14 (24). $\times 1\frac{1}{2}$.



15 (23). $\times 5$.



16 (27). $\times 5$.

the specimens, taken individually, at first sight give the impression of Crustacean affinities. But a consideration of all the specimens seems to me to render this view quite untenable. The impression that the organism was a Crustacean evidently depends chiefly upon the assumptions that the "capsule" was a carapace, that the "primary segmented rod" was the jointed body of the animal, and that the "lobes" and the intermediate structures between them and the primary rod were the appendages, probably foliaceous in character. But there are many facts against these assumptions. For instance:

(1) If the "capsule" were indeed a carapace it must have been open round much of its margin, as is shown by the protrusion of the supposed body. But in that case it should surely have shown, in some at least of

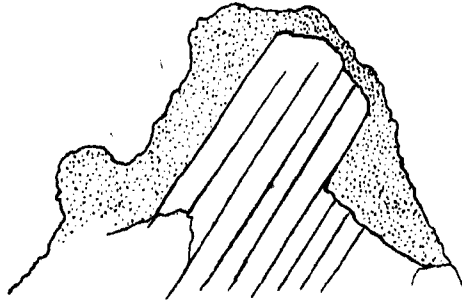


FIG. 1—Small portion of the "cuticle" showing a clear striated layer overlying a finely granular layer. $\times 250$.

the 25 specimens in which it occurs, some displacement of the free edges so that they would not always exactly coincide and give the appearance of a bag-like structure. Actually, however, all the specimens do give this impression of a closed and probably two-walled sac. Again, the truncated end of the "capsule", with its "palisade" and "cirri", seems to be an impossible anterior termination of a carapace.

(2) If the "primary segmented rod" were the body of the animal it ought to show some evidence of its presence under the anterior and middle parts of the capsule. Actually, wherever it can be seen, it always commences abruptly near the edge of the capsule. Again, it always projects from the capsule at a well-marked angle, usually nearly a right angle, without showing any sign of curvature, which it should do if it had been a flexible body. There is also difficulty about the spines. Although a median row of spines can, of course, occur on the back of a Crustacean, I do not think there is any known instance where they are bifurcated at

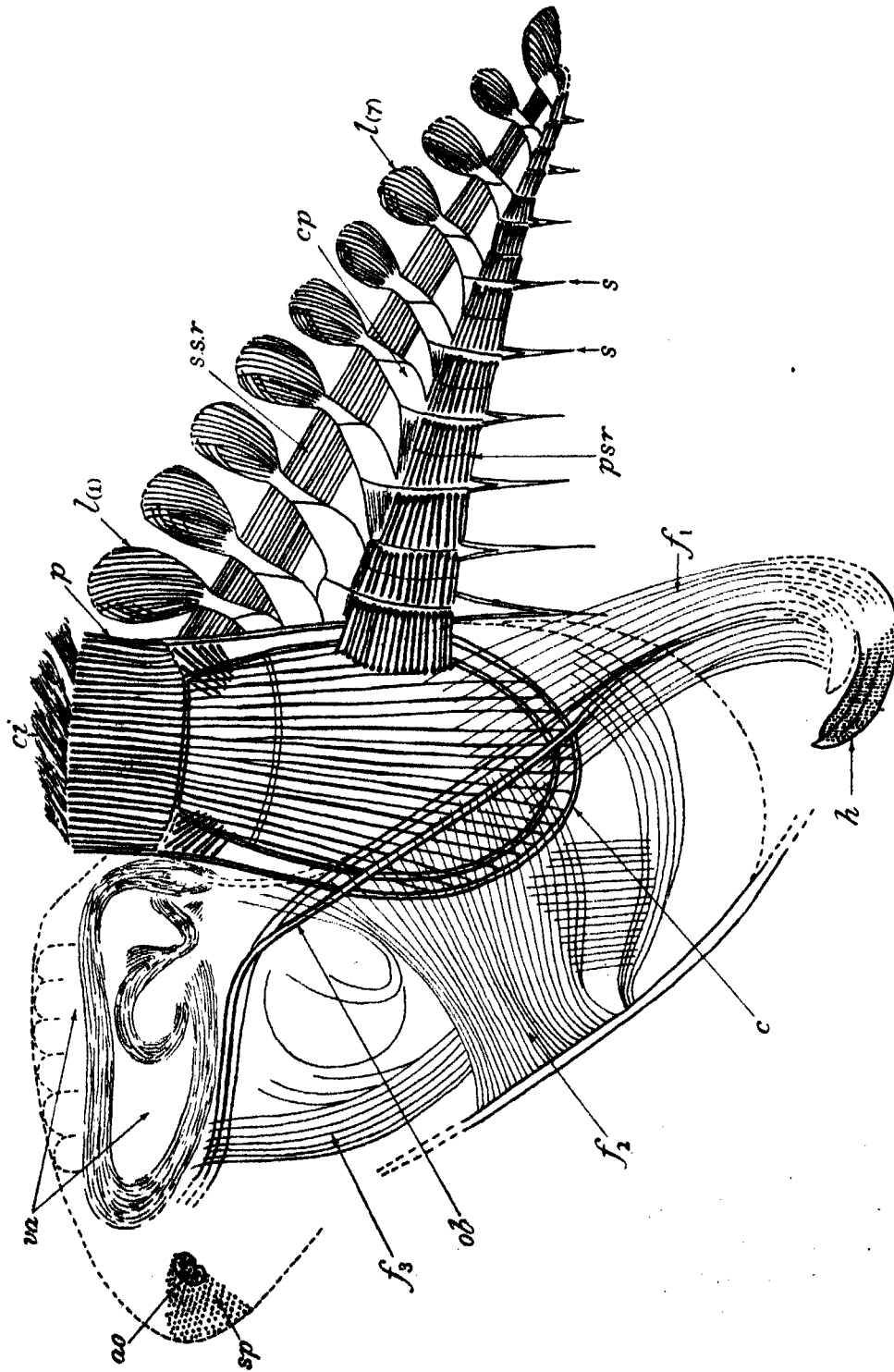


Fig. 2.

- | | | |
|------------------------|----------------------------------|-------------------------------|
| ao, auriculate organ. | f ₁ , fascicle No. 1. | s, spines. |
| c, capsule. | f ₂ , fascicle No. 2. | sp, shagreen patch. |
| ci, cirri. | f ₃ , fascicle No. 3. | ssr, secondary segmented rod. |
| cp, connecting pieces. | h, hook. | va, vague area. |
| | l, lobes. | |
| | ob, oblique bands. | |
| | p, palisade. | |
| | psr, primary segmented rod. | |

the base. Further, the longest spine is quite close to the capsule, and it is most improbable that such a spine should have been developed *under* a carapace, for that would have been its position when the anterior part of the supposed body was in its normal position, namely, covered by the carapace.

(3) If the "lobes", etc., were really appendages, it is almost certain that they would have shown somewhere that they were paired structures. Actually there seems no evidence that they were bilateral organs, but rather that they formed a single median row.

In addition to these difficulties in correlating the capsule, primary rod, and lobes with a carapace, body, and paired appendages, there are all the other structures on and around the capsule to be taken into account, such as the "oblique bands", the "hook", and the "fascicles". How can all these be worked into any scheme of Crustacean morphology? Or, indeed, into any scheme of Arthropod morphology?

Assuming, therefore, that the organism was not an Arthropod, to which of the other animal phyla could it have belonged? The segmented character of part of its organization seems to rule out the possibility of such groups as the Coelenterata, Echinodermata, and Mollusca, and, in fact, all the other phyla except the Chaetopoda and the Chordata. As it seems quite impossible to see in it any Chaetopod affinities, it remains to consider whether it could possibly have been a Chordate.

It is fairly obvious, of course, that if it were a Chordate it could not have been of a very advanced type, apparently not up to the level of even the lowest of the fishes, since there seems to be no indication of anything that could properly be called a head. The problem therefore resolves itself into whether there is any way of looking at the organism which would bring it into some sort of harmony with any of the lower Chordate groups. It seems to me that there is such a way and, further, that it is the only way. It is to assume that the organism obtained its food by filtering small particles out of the water and that the "capsule" was the filtering chamber. The capsule would then become the homologue of the pharynx in the Tunicates and the "palisade" and the "cirri" would fall into line as structures surrounding the opening into its interior, while the longitudinal and slightly radiating lines on its surface might reasonably be interpreted as strengthening fibres (? cartilaginous) in its presumably perforated walls.

Assuming for the moment, therefore, that the capsule was the equivalent of the pharynx in the Tunicates, the question at once arises whether any of the other structures fit in with what is known of Tunicate morphology, either adult or larval. As to this I think the "segmented rods" with

their spines and "lobes" and intermediate parts, which, as already mentioned, probably formed a single whole, correspond fairly closely, so far as position in relation to the capsule is concerned, to the "tail" in the tadpole larva of many Tunicates. It is true that if these structures really formed a tail it was a much more complex organ than anything found at any stage in recent Tunicates. But the Tunicates are admittedly degenerate forms, and there seems good *prima facie* reason to suppose that they come from a stock possessing more fully developed characters, especially as regards powers of locomotion. Moreover, the plan underlying this complexity of structure is not inconsistent with Chordate development, very much the reverse, in fact, for its elaboration on vertebrate lines seems almost too obvious to be true.

It appears, therefore, that as regards two important structures the organism might conceivably have been related to earlier and in some respects more highly evolved Tunicates. As regards the remaining structures that have been preserved in the specimens, it seems impossible to speak with any confidence. The thin material overlying the black carbon in many places, previously referred to as the "cuticle", may possibly represent an enveloping test somewhat similar to that of modern Tunicates, and the "hook" and other structures may have been in the main arrangements for the fixation or anchoring of the organism, although in view of the strongly developed "tail" this seems debatable. Such an organ seems rather to suggest a locomotory habit, and as it seems to have been equally well developed in all specimens it could scarcely have been a larval character destined to atrophy as the organism grew older and adopted a different mode of life.

No doubt there are many difficulties in the way of accepting the view that the organism was related to the Tunicates. One of these obviously concerns the original nature of the material which has been preserved in the specimens. What was it? No recent Tunicate seems to have any parts sufficiently firm to allow of fossilization in the very definite form in which it is found in many of the specimens, especially considering the matrix in which they are preserved, which is by no means of the finest. Although it is very unsafe to set limits to what may be fossilized, one would certainly think that the structures preserved in this case must have been of considerable consistency, say, at least equal to cartilage as a rule and sometimes very much firmer, as in the case of the "hook" with its extremely fine cross-ridges. Whether this is a valid argument against the possibility of Tunicate relationship is, however, probably a matter of opinion. Another difficulty is the high degree of specialization of the "tail". If an early type of Tunicate could have had such an elaborate

organ why do we not find more distinct traces of it somewhere in the group at the present day? Yet another difficulty, of course, is the apparent impossibility of correlating the many structures other than the capsule and the segmented rods with any of those found in recent Tunicates.

Such difficulties may prove insuperable, but, in the meantime, I am disposed to consider the organism as at least allied to the Tunicates. If the possibility of Arthropod affinity is ruled out, as I believe it must be, there seems no way in which the organism can be made intelligible, made to work so to speak, except by regarding it as a Chordate allied to the Urochorda.

Nevertheless, it is much to be desired that further specimens should be obtained so that any ambiguity as to its true nature may be finally dispelled.

As a name is necessary, I propose to call the organism *Ainiktozoon* loganense* gen. et sp. nov., assuming for the time being that we are only dealing with one species. The holotype is specimen No. 32, shown in photograph No. 1, Plate 18.

My sincere thanks are due to all those who have shown interest in the matter and offered suggestions during the course of my study of these specimens, and especially to Professor E. B. Bailey, Dr. W. T. Calman, Mr. W. Fletcher, Professor W. Garstang, Mr. D. M. Reid, Dr. H. Dighton Thomas, and Professor J. Walton for much valuable help in various ways.

Since the above paper was written, the specimens referred to therein by their original temporary numbers have received official registration numbers, partly at the Hunterian Museum, Glasgow, and partly at the British Museum (Natural History). The list of the corresponding numbers is as follows :

Temporary numbers quoted in paper	Registration numbers		Photograph number
	Hunterian Museum	British Museum (Nat. Hist.)	
1	V 6490	—	11
2	V 6491	—	8
3	—	In. 34963	—
4	V 6492	—	12
5	V 6493	—	5
6	V 6505	—	—
7	V 6494	—	7
8	—	In. 34964	—
9	V 6506	—	—

* From *ainiktos* = riddling, enigmatical.

Temporary number quoted in paper	Registration numbers		Photograph number
	Hunterian Museum	British Museum (Nat. Hist.)	
10	V 6495		10
11	V 6507	—	—
13	—	In. 34965	—
14	V 6508	—	—
15	V 6509	—	—
16	—	In. 34966	—
17	V 6510	—	—
18	V 6511	—	—
19	V 6496	—	4
20	—	In. 34967	—
21	V 6497	—	9
23	V 6499	—	3 + 15
24	V 6500	—	14
25	—	In. 34968	—
26	V 6501	—	2
27	V 6502	—	6 + 16
28	V 6498	—	—
29	—	In. 34969	—
30	V 6503	—	13
32	V 6504	—	1

SUMMARY

A detailed study has been made of a series of specimens of a peculiar fossil organism from the Ludlow Beds of Logan Water, Lesmahagow. They were collected about 1904, but have not previously been figured and described, except for a short notice (Scourfield, 1929-30). The specimens evidently represent the same type of organism, but a reconstruction which has been made embodying all justifiable details does not give an unequivocal answer as to the phylum to which it belonged. The two most important features are a central ovoid body, ranging from about one to nearly three inches in length, referred to in the paper as the "capsule", and two converging segmented rods from one of which arise spines with bifurcated bases and from the other a series of lobes. There seems no reason for supposing that the organism was a plant, and arguments are brought forward which, it is believed, preclude the idea that it was a Crustacean or any sort of Arthropod or even any known type of Invertebrate. As to the possibility of its being a Chordate, it is pointed out that if it be assumed that the animal obtained its food by filtering small particles out of the water and that the "capsule" was the filtering

chamber, then the segmented rods could be regarded as the elaborated homologue of the "tail" of the tadpole larva of many Tunicates. Consequently, in spite of the fact that its other structures cannot at present be correlated with those found in recent Tunicates, it does seem possible, provisionally at any rate, to regard the organism as a Chordate of lowly type allied to the Urochorda.

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Anthraquinone Pigments in Galium

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(Communicated by Sir Frederick Hopkins, F.R.S.—Received 29 September, 1936)

INTRODUCTION

The occurrence of chemical as distinct from morphological varieties has been shown in the plant *Eucalyptus Dives* by Penfold and Morrison (1928). An example of definite chemical variation in animals is that of the Dalmatian hound, where the excretion of uric acid peculiar to this variety was shown by Onslow (1923) to behave genetically in the same way as a morphological character. More recently Scott-Moncrieff (1936) and Lawrence and Scott-Moncrieff (1935), who have extended the earlier investigations of Mrs. Onslow (1925) on the genetics of the occurrence of the anthocyanins, which give the colours of flowers and fruits, have shown that the study of chemical genetics can give a clue to processes taking place in the organism. This work forms the foundations for the development of the branch of biology—Chemical Variation. While, for example, a certain terpene may characterize a species, in some cases a chemical substance may characterize a whole genus or group of genera. The last consideration concerns the present work.

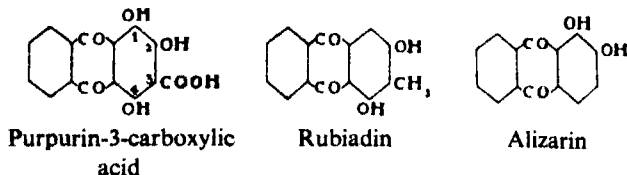
Anthocyanins and substances related to them have been found by Robinson and Robinson (1934) to be almost general in the plant kingdom. The knowledge of hydroxyanthraquinones in plants, the substances with which the present paper is concerned, has recently received two important additions—the work of Raistrick, Robinson, and Todd (1934) on the Mould *Helminthosporum* and of Kögl and Deijs (1934) on the Toadstool *Boletus*. Previously, hydroxyanthraquinones were known in Lichens and in Angiosperms; in *Rhammus* and *Ventilago* (Rhamnaceae), in *Rumex* and *Polygonum*; in *Cassia* and *Andira* (Leguminosae); in *Aloe* (Liliaceae) as well as in Madder and several other members of the Rubiaceae. The anthraquinone structure occurs in isolated cases throughout the plant kingdom, which suggests that, like the anthocyanidin and anthoxanthin structure, it may be the end product of a general type of metabolism leading to aromatic compounds.

In the present work the tribe *Galieae* (or *Stellateae*) of the Rubiaceae has been chosen for a survey because all its species so far examined have been found to contain glycosides of hydroxyanthraquinones, the object being to see if these plants were suitable for a study of chemical variation. This group contains the madder plant *Rubia tinctorum* L., of which much of the chemistry is already known because of its use formerly in dyeing. Examination of the pigments of the related Bedstraws (*Galium*) has yielded the results described here which have added to the knowledge of the pigments of madder itself. It has been found that the colouring matter alizarin (the most valuable constituent of madder) is subject to great variation in amount in species and in strains of one species, and that purpurin (the less valuable dye of madder) occurs as its carboxylic acid in the whole tribe *Galieae*, and is subject to less variation. Purpurin, which is present in the dried madder root, is not present in the fresh plant, but is derived from the purpurin-3-carboxylic acid originally present. This latter substance (which is isomeric with the pigment boletol obtained from *Boletus* by Kögl and Deijs) occurs as a glycoside which has now been isolated. The name *galiosin* is proposed for this glycoside of purpurin-3-carboxylic acid because it is a substance which has been found only in the tribe *Galieae* of the Rubiaceae and appears to be characteristic of it.

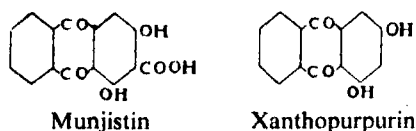
THE AGLYCONES AND METHODS OF ESTIMATION

The three principal aglycone pigments which concern the present work are purpurin-3-carboxylic acid (1:2:4-trihydroxyanthraquinone-3-carboxylic acid), rubiadin (2:4-dihydroxy-3-methyl-anthraquinone), and

alizarin (1:2-dihydroxyanthraquinone). A summary of the literature is given by Perkin and Everest (1918).



These three pigments are present in the plants as glycosides. The glycoside of alizarin, ruberythric acid, was isolated from madder by Rochleder (1851). By the reduction of purpurin-3-carboxylic acid or galiosin, its glycoside, there is formed munjistin or 2:4-dihydroxyanthraquinone-3-carboxylic acid. This last substance has been isolated from madder and occurs in larger quantities in *Rubia munjista*. Loss of CO_2 gives xanthopurpurin, which has also been isolated from the dried madder root.



METHODS OF ESTIMATING PURPURIN-3-CARBOXYLIC ACID AND ALIZARIN

Purpurin-3-carboxylic acid and alizarin are particularly suitable substances for quantitative estimation since they both possess well-defined and characteristic absorption spectra. Purpurin-3-carboxylic acid shows very distinct bands in toluene solution, by which it may be readily distinguished from purpurin (fig. 1). Alizarin shows a well-defined absorption spectrum in alkali. Rubiadin gives a red solution in alkali without clear bands. Two methods of estimation were used:—

(a) The clean fresh roots (0.5–2 gm.) were weighed, put into boiling water for 5 minutes, then extracted with water until the yellow colour had all been extracted, crushing if necessary. To the clear filtered solution 0.5 to 1% of HCl was added and 30 cc. of toluene. The mixture was warmed for $\frac{1}{2}$ hour to 50°C . and the purpurin-3-carboxylic acid resulting from the hydrolysis of galiosin extracted with toluene and the red toluene solution made up to a known volume (50 or 100 cc.). A layer of the solution 1 cm. thick was then compared in a spectrophotometer with a standard solution of purpurin-3-carboxylic acid (0.02 mg. per cc.)

in toluene. The remaining aqueous solution of the other glycosides was then boiled with 10% HCl for 20 minutes, extracted with toluene, and the pale yellow toluene solution made up to a known volume. A measured volume of this was then extracted with a measured volume of 0.5% NaOH and the alkaline solution of alizarin and rubiadin obtained compared with a standard solution of alizarin (0.02 mg. per cc.) in 0.5% NaOH in a spectrophotometer. The presence of rubiadin does not interfere with the estimation of alizarin. The absorption band in the orange region (6300 Å.) was used. The alizarin was then removed by precipitation with baryta, and the colour of the orange red solution of rubiadin compared with

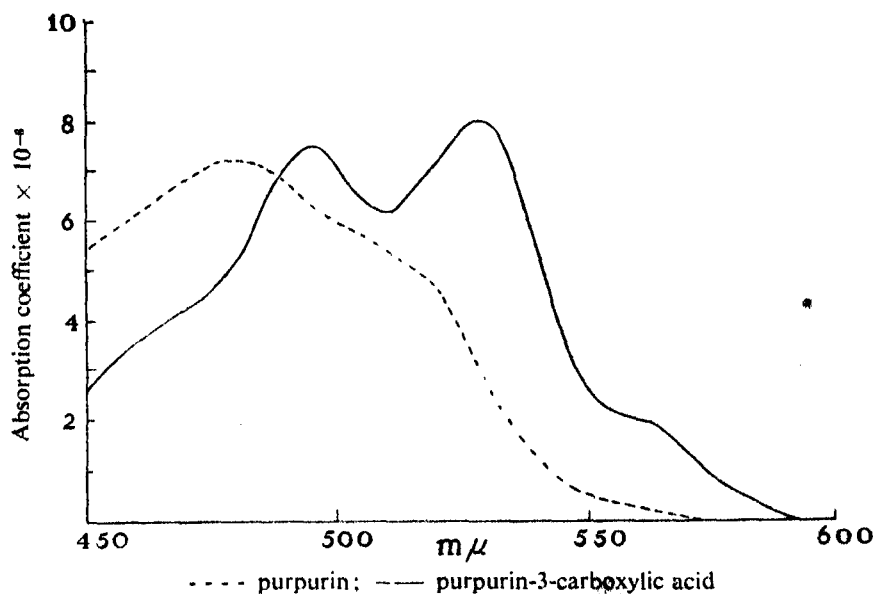


FIG. 1—Absorption spectra of purpurin and purpurin-3-carboxylic acid in toluene.

standard solutions of rubiadin in 0.5% NaOH in test tubes. This gave an approximate measure of the amount of rubiadin, but the colour match was not good owing to impurities.

(b) The cleaned fresh roots were cut up, weighed, and put into a flask containing 100 cc. of toluene and 20 cc. of 10% HCl. The flask was shaken at intervals and left for two days. It was then heated to 100° C. on a water bath for 25 minutes. The glycosides were thus decomposed and the pigments passed into the toluene. The purpurin-3-carboxylic acid was then estimated as before in the toluene, the presence of the other pigments not interfering. The solution was extracted with NaHCO_3 or neutral sodium tartrate, which left the alizarin and rubiadin

as a pale yellow solution. The estimation was then continued as previously described.

DISTRIBUTION OF PURPURIN-3-CARBOXYLIC ACID AND ALIZARIN IN SPECIES

Purpurin-3-carboxylic acid was found to be constantly present in the roots of members of the tribe (*Galieae*) of the Rubiaceae. Alizarin was present in significant amounts only in certain species or strains in this group. While a specimen of the cultivated madder plant *Rubia tinctorum* (from the Botanical Garden, Cambridge) contained about equal amounts of alizarin and purpurin-3-carboxylic acid specimens of the wild madder *R. peregrina* (from Dorsetshire) contained no alizarin but a large amount of purpurin-3-carboxylic acid. Alizarin was present in *Galium mollugo* and especially in *G. erectum* and the intermediate types. In *G. verum* alizarin was found only in traces. A natural hybrid *G. verum* $\times$ *erectum* gave in the self-pollinated F_2 generation a great variation in the relative proportions of alizarin and purpurin-3-carboxylic acid. Fig. 2 shows the

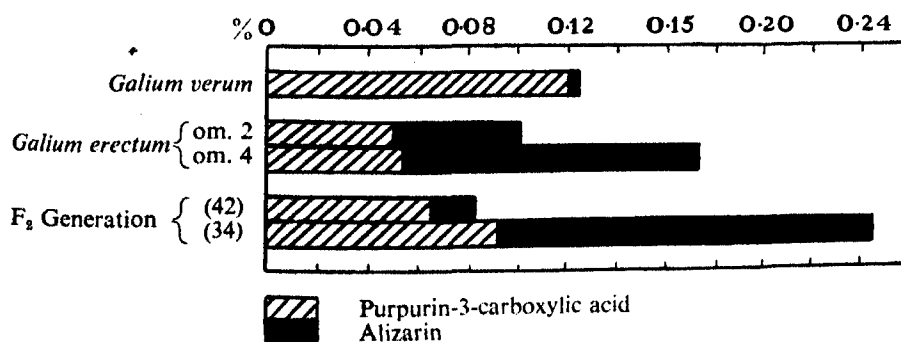


FIG. 2.

proportions of alizarin and purpurin-3-carboxylic acid represented as a percentage of the fresh weight of the roots in (a) *G. verum* and (b) limits of variation in *G. erectum* from the locality of the hybrid, (c) limits of variation in 39 of the F_2 generation. Fig. 3 shows frequency polygons for the percentage of alizarin and of purpurin-3-carboxylic acid in the 39 individuals. It can be seen that the amount of purpurin-3-carboxylic acid was more constant than that of alizarin. The flower colour of the 39 F_2 generation was all white while the original hybrid *G. verum* $\times$ *erectum* had pale yellow flowers intermediate between the two species. It has since been found that plants can occur in the F_2 generation with yellow flowers, but these appear only under careful conditions of cultiva-

tion, being less vigorous than the plants with white flowers. On continued inbreeding the hybrid gradually appears to give normal *G. verum* and *G. erectum*. The work is being continued to see if the alizarin specifically appears in the resulting *G. erectum* or whether it will appear now in the *G. verum*. Further breeding is in progress to see if it is possible to obtain comparatively pure strains showing constant chemical and morphological differences. The results given here show that there is a possibility of this being realized, especially as the chemical differences have been found to persist throughout the year.

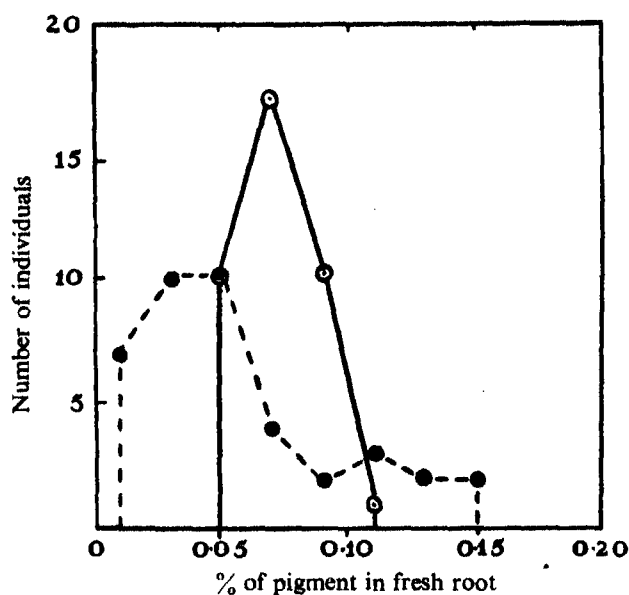


FIG. 3.—Frequency polygons for alizarin and purpurin-3-carboxylic acid.

○ Purpurin-3-carboxylic acid; ● alizarin.

In Table I estimations of purpurin-3-carboxylic acid in a number of species are shown. *Galium saxatile* and the allied species *G. sylvestre* were found to contain very little purpurin-3-carboxylic acid, and no variety of either species has been found up to the present having root phloem of the distinct yellow colour found in other species. It must be emphasized here that purpurin-3-carboxylic acid has not been found so far in any other group of plants or in other genera of the Rubiaceae. Among the species in which alizarin is present may be mentioned *Asperula ciliata*, *Galium rubioides*, *G. sylvaticum*.

For a study of chemical variation the knowledge of the chemical structure of the glycosides actually present in the plant must be as accurate

as possible. The separation and isolation of the hydroxyanthraquinone glycosides concerned in the present work have therefore been investigated, and the results are described here. The determination of their chemical constitutions is being described in full elsewhere (Hill and Richter, 1936; Richter, 1936).

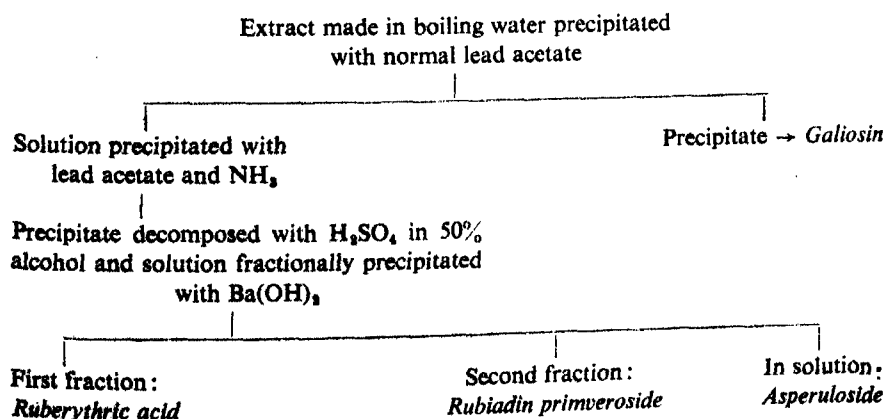
TABLE I—% PURPURIN-3-CARBOXYLIC ACID FOUND IN FRESH ROOTS

<i>Asperula odorata</i>	0.08	<i>Galium rubioides</i>	0.13
„ <i>ciliata</i>	0.13	„ <i>rubrum</i>	0.08
„ <i>cynanchica</i>	0.06	„ <i>saxatile</i>	0.03
„ <i>hirta</i>	0.12	„ <i>sylvaticum</i>	0.09
<i>Crucianella stylosa</i>	0.20	„ <i>sylvestre</i>	0.02
<i>Galium anglicum</i>	0.04	„ <i>tricornis</i>	0.08
„ <i>aparine</i>	0.05	„ <i>uliginosum</i>	0.11
„ <i>boreale</i>	0.12	„ <i>verum</i>	0.15
„ <i>cruciata</i>	0.08	<i>Rubia peregrina</i>	0.25
„ <i>erectum</i>	0.07	„ <i>tinctorum</i>	0.17
„ <i>mollugo</i>	0.08	<i>Sherardia arvensis</i>	0.08
„ <i>palustre</i>	0.21		

SEPARATION OF THE GLYCOSIDES PRESENT IN *Galium*, ETC.

The methods used in the present work for isolating galiosin, ruberythric acid, and rubiadin primveroside from different members of the *Galieae* were less drastic than the methods which have generally been employed. The enzymes were destroyed and the glycosides extracted by introducing the roots for a short time into boiling water, which was satisfactory if the

TABLE II



material was in a finely divided condition. The glycosides were then separated by precipitation with lead acetate followed by fractional precipitation with barium hydroxide in 50% alcoholic solution. The separation in the precipitation with barium hydroxide was remarkably sharp.

The fractional precipitations were controlled by hydrolysing samples with acid and examining the absorption spectra of the aglycones. The asperuloside was recognized by the green colour on boiling with acid (Hérissey, 1926). It seems probable that these glycosides exist in the living plants.

Galiosin—The fresh roots of *Rubia tinctorum* and of most *Galiums* are yellow in colour, but on bruising they rapidly turn bright red. This does not take place if the roots are first immersed in boiling water. This change in colour was found not to be due to an oxidation such as occurs in the darkening in *Vicia faba* and other plant but to the rapid enzymic hydrolysis of a yellow glycoside. The yellow substance was isolated and found to be a glycoside of purpurin-3-carboxylic acid, the reddening of the bruised tissues of the plant being due to the liberation of the red aglycone.

This yellow glycoside, which was given the name Galiosin, was extremely unstable. It was decomposed, for example, on standing with cold dilute HCl, with Na_2CO_3 solution or by boiling for a few hours with either alcohol or distilled water.

Isolation of Galiosin from Rubia tinctorum—1245 gm. of fresh madder root were washed and cut into small pieces, care being taken to bruise the root as little as possible. The material was then added to 5 litres of boiling water, boiled for 17 minutes, and the mixture cooled as rapidly as possible. To the fluid kieselguhr was added to ensure rapid filtration. To the clear filtrate 300 cc. of 25% normal lead acetate were added and the dark reddish-brown precipitate filtered through a bed of kieselguhr. In this filtration, as in all the subsequent filtrations described below, an amount of kieselguhr approximately equal in bulk to the precipitate was stirred into the solution before filtration, and the mixture of precipitate and kieselguhr was filtered and washed together. The precipitate containing most of the galiosin was stirred with distilled water to a smooth paste and shaken in a mechanical shaker for 30 minutes with 500 cc. 3% acetic acid. The mixture was then filtered and the galiosin washed out with a further 100 cc. of 3% acetic acid containing 1% lead acetate. The deep yellow solution was treated with 30 cc. of 25% lead acetate, the

precipitate discarded, and the glycosides precipitated as lead salts by adding 3% ammonia until a test with Brom Thymol Blue indicated p_H 7.5. The red precipitate, after washing with water, was dissolved in 1% acetic acid, treated with 25% normal lead acetate until there was no further precipitation, the precipitate discarded, and the lead salts of the glycosides again precipitated by ammonia. The precipitate was now decomposed by shaking with 1% sulphuric acid at 0° C.; the solution became yellow and sulphuric acid was added until it was faintly acid to Congo Red. The yellow solution was now neutralized to p_H 7 (Brom Thymol Blue) with baryta solution. After filtering, the non-acidic glycosides were removed by several extractions with butyl alcohol. The aqueous solution was then made acid to Congo Red with 2% sulphuric acid and the galiosin repeatedly extracted with a large volume of butyl alcohol. The galiosin was extracted again from the butyl alcohol with 5 cc. of 3% ammonium carbonate solution, and the extract put in a shallow dish on a water bath while the ammonia and water were blown off by a rapid current of air. On adding a little water to the residue and evaporating rapidly, as before, the glycoside separated as shimmering yellow needles. It was recrystallized from a small volume of water at 60° C. Yield 0.7 gm.

In further purification for analysis the aqueous solution containing 3.5% of galiosin was treated with an equal volume of methyl alcohol and left at 0° C. The crystals were washed with 50% methyl alcohol and finally with ether.

Galiosin was isolated in a similar manner from *Galium verum*, *Galium mollugo*, and *Rubia peregrina*. The colour reactions characteristic of galiosin were given by the other species from which purpurin-3-carboxylic acid was obtained. In samples of commercial madder root, little or no galiosin was present, the purpurin carboxylic acid being present in the free state.

Rubiadin primveroside—The roots of *Galium verum* contained very little alizarin glycoside, but in addition to galiosin they contained a considerable amount of a glycoside which had the properties of a rubiadin primveroside.

Preparation of rubiadin primveroside—500 gm. of the fresh washed roots of *G. verum* were cut into small pieces and extracted with boiling water, as in the preparation of galiosin. After the first precipitation with normal lead acetate which removes the galiosin, the clear filtrate was treated with lead acetate and ammonia (p_H 7.5 Brom Thymol Blue).

The resulting precipitate was decomposed with sulphuric acid, the lead sulphate removed, and the glycosides diluted to 250 cc. in 40% alcohol. Ba(OH)_2 solution was added until the precipitate, at first brownish-white, became slightly pink. The mixture was filtered and more Ba(OH)_2 solution added so long as a dark crimson precipitate was formed. This was filtered off and decomposed with sulphuric acid, and the precipitation repeated in 70% alcohol. The barium salts were then decomposed with sulphuric acid and the yellow solution of glycosides evaporated down to a small volume (10 cc.) and poured with shaking into 300 cc. of 97% alcohol. The precipitate containing phenolic glycosides was discarded and the alcoholic solution evaporated to a small volume. On standing for several days the yellow rubiadin primveroside crystallized out; subsequently the crystallization could be aided by seeding. After three to four recrystallizations from 50% alcohol the rubiadin primveroside melted at 248–250° C. Yield 0.7 gm. (i.e., 0.14% of the fresh root). The glycoside was prepared in a similar manner from *Galium mollugo*.

Preparation of Ruberythric Acid from Rubia tinctorum—1245 gm. of fresh madder root were treated as described under the preparation of galiosin. After the first precipitation with normal lead acetate ammonia was added to the clear yellow solution until it was slightly alkaline (blue with Brom Thymol Blue). Kieselguhr was added to the mixture and the red precipitate filtered off and washed with water. The precipitate, containing the glycosides and other substances, was stirred into a paste with water, and 10% sulphuric acid was stirred in until the mixture was just acid to Congo Red. To the yellow solution (310 cc.) obtained by filtration an equal volume of alcohol was added and the solution again filtered. Barium hydroxide was then added very slowly with vigorous stirring. When a small sample of the solution obtained by filtering gave with more barium hydroxide a red precipitate which was found on hydrolysis to give no spectroscopic test for alizarin, the whole was filtered. The ruberythric acid was precipitated completely in this first fraction.

The barium salt of ruberythric acid was decomposed with dilute sulphuric acid, and the crystalline precipitate of ruberythric acid together with barium sulphate was washed with chloroform to remove any impurity. The glycoside then was extracted with boiling water, from which it crystallized on cooling. Yield 2.5 gm. (0.2% of the root), m.p. 257° C.

SOME OBSERVATIONS ON THE ENZYMIC HYDROLYSIS OF HYDROXY- ANTHRAQUINONE PRIMVEROSIDES

The three glycosides galiosin, ruberythric acid, and rubiadin primveroside have been found to be derived from the same disaccharide, primverose, or 6-(β -D-xylosido)-D-glucose (Hill and Richter, 1936; Richter, 1936). Since rubiadin primveroside readily gave rubiadin-3-glucoside and xylose on mild hydrolysis, and pure primverose was isolated from the other two glycosides, the glucose moiety of the disaccharide is in each case attached to the aglycone. In rubiadin primveroside and ruberythric acid the sugar residues are on the 2- (or β -) hydroxyl groups of the anthraquinone nucleus, while in galiosin it appeared to be in the 1- (or α -) position, which accounts for the ready hydrolysis of this last glycoside.

The enzyme erythrozym which is present in the madder root and is responsible for the hydrolysis of ruberythric acid to alizarin was one of the first enzymes to be investigated (Schunck, 1854). In view of the identification of the sugar of ruberythric acid as primverose, erythrozym must belong to the class of "primverosidases" which occur in the Primulaceae (Bridel, 1925); but the nature of the specificity of the glycosidases is so little understood that there is little justification for the definition of a separate class of "primverosidases".

It is clear from the present work that certain plants which contain active glycosidases will not appreciably hydrolyse the glycosides of madder, while in nearly all cases tried primveroside containing plants readily hydrolysed them. Galiosin was generally hydrolysed more easily than ruberythric acid or rubiadin primveroside. The hydrolysis of galiosin by an appropriate enzyme was in many cases very rapid (20 minutes), and the colour change to red when purpurin-carboxylic acid was liberated from the yellow galiosin makes a convenient test for rapid work.

The hydrolysis of the glycosides was followed by extracting the aglycones with toluene or chloroform. With ruberythric acid and rubiadin primveroside the aglycones were estimated colorimetrically in the solution obtained by extracting the chloroform solution with sodium hydroxide.

The plant extracts were prepared by grinding in a mortar with water: the suspension was then strained through muslin before use. A control experiment was always carried out at the same time by incubating the glycoside with a boiled suspension. In long incubations toluene was added as an antiseptic, but for shorter periods this was not considered necessary.

In Tables III and IV a qualitative survey of certain plants is shown: in the hydrolysis of the three glycosides, + indicates nearly complete hydrolysis, — no perceptible hydrolysis, ± slight hydrolysis.

TABLE III—HYDROLYSIS OF GALIOSIN. IN EACH EXPERIMENT 0.005 MG. OF GALIOSIN IN 3 CC.

Species	Hydrolysis	Time of incubation at 18° C. mins.
<i>Galium verum</i> (root)	+	20
<i>Geum urbanum</i> (root)	—	1400
<i>Hottonia palustris</i> (stem)	+	93
<i>Primula officinalis</i> (bud)	+	20
<i>Primula elatior</i> (root)	+	30
<i>Primula sinensis</i> (petiole)	+	150
<i>Primula vulgaris</i> (root)	+	20
<i>Rhamnus catharticus</i> (stem bark)	+	60
<i>Rubia peregrina</i> (root)	+	20
<i>Rubia tinctorum</i> (root)	±	20
<i>Spiraea ulmifolia</i> (bud)	—	780
<i>Vicia faba</i> (leaf)	±	600

TABLE IV—HYDROLYSIS OF RUBERYTHRIC ACID AND RUBIADIN PRIMVEROSIDE. IN EACH EXPERIMENT 1 CC. PLANT EXTRACT + 0.005 MG. GLYCOSIDE IN 2 CC. WATER; INCUBATED AT 37° C.

Species	Ruberythric acid mins.	Rubiadin primveroside mins.
<i>Primula officinalis</i> (root)	+ 360	+ 360
<i>Primula elatior</i> (root)	+ 240	+ 240
<i>Primula sinensis</i> (petiole)	+ 720	+ 720
<i>Rubia tinctorum</i> (root)	+ 720	+ 720

DISCUSSION

The substance purpurin-3-carboxylic acid was found in all the 23 species (including 5 genera) that were examined of the genus *Galieae* of the Rubiaceae. Since it has not been found in any other group of plants or in any other genus of the Rubiaceae, purpurin-3-carboxylic acid can be considered as a chemical character for the genus unless numerous exceptions are found when the observations are extended. Rubiadin also has not been found outside this group and may be general to it, while alizarin, which cannot be detected in some species, has been found also in the medicinal Rhubarb (Polygonaceae), but only in minute amounts.

It was observed that no alizarin was present in the young and etiolated stems of the madder plant, while galiosin, the primveroside of purpurin-3-carboxylic acid, was abundantly present. Again in varieties of *Galium erectum* containing alizarin, it was found that the alizarin was mainly in the outer part of the root phloem; in the inner part galiosin and rubiadin primveroside were present. It appears, then, that alizarin may be a later product of metabolism.

Galiosin on reduction in neutral aqueous solution with hydrogen and palladium at 18° C. gave munjistin. In a sample of *Rubia* sp. from Sikkim (possibly *Rubia sikkimensis*) containing unhydrolysed galiosin it was found that considerable quantities of free munjistin were present, but no munjistin glycoside could be found. This suggests that munjistin may arise as a natural reduction product of galiosin in the plant. If the carboxyl group of munjistin were replaced by a methyl group, rubiadin would result. In the rubiadin primveroside and in ruberythric acid the sugar is on the 2- (or β -) hydroxyl group, while in galiosin it appeared to be in position 1- (or α). If the sugar occurs on different positions in the hydroxyanthraquinones of the same plant it would seem that in this case transformations of the aglycones may take place previously to the final glycoside formation.

The rapid hydrolysis of these hydroxyanthraquinone primverosides by enzymes from species of *Primula* suggests that the specificity of certain glycosidases is determined by the sugar. *Geum urhanum*, which contains an active enzyme hydrolysing a vicianoside of eugenol, was without action on galiosin. Certain plants, however, were found which selectively hydrolysed ruberythric acid more rapidly than the less stable galiosin, showing alternatively an influence of the aglycone.

We are indebted to Sir Frederick Hopkins for his interest in this work and wish to thank Mr. Gilbert-Carter for his help in obtaining many of the plants, and also Mr. T. Tutin for sending us roots of *Galium boreale*. Nearly all the species examined we have under cultivation and we are very grateful to Dr. J. Needham and Dr. A. Walton for placing ground at our disposal.

SUMMARY

Two glycosides, identified as a primveroside of purpurin-3-carboxylic acid (Galiosin) and a primveroside of rubiadin, have been found in species of *Galium* and *Rubia*.

Purpurin-3-carboxylic acid may be typical of the genus *Galieae* of Rubiaceae.

Convenient methods of separation and isolation of the glycosides, galiosin, ruberythric acid, and rubiadin primveroside are described.

The hydroxyanthraquinone glycosides from *Galium* and *Rubia* are hydrolysed by enzymes present in species of *Primula*.

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Observations on the Parasitism and Control of *Armillaria mellea*

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[PLATES 23–25]

1—INTRODUCTION

Armillaria root disease has been known since 1874. Until recently, the fungus was believed to attack roots only through wounds. Thomas (1934), however, has shown in an interesting paper that *Armillaria mellea* (Vahl.) Quel, penetrates the roots of healthy plants in the absence of wounds. His paper gives such a full review of the literature connected with the parasitism of this fungus that repetition would be superfluous here. Thomas gives a detailed description of the entry of *Armillaria* rhizomorphs into various roots and tubers. He compared the reaction in the tissues of resistant and susceptible plants but, unfortunately, could not formulate any control measures as a result of his observations. Wallace (1935) has reviewed the control measures employed up to date against the disease.

Armillaria, however, has caused, and is still causing, serious losses to tea in Nyasaland, although thorough stumping and root clearing have been employed as much as is practicable and economic in the opening up of new tea gardens. The author has been forced to the conclusion that control measures as advocated up to date are very far from satisfactory.

The present work is the outcome of what was originally an attempt to obtain infection of tea seedlings in the hope that a method might be found to induce resistance in the seedlings. The infection of tea seedlings has been obtained by a method somewhat similar to that used by Thomas. Examination of the infected seedlings, however, suggested an hypothesis for control of the disease on entirely new lines, so that the original idea of inducing resistance was rejected.

The hypothesis has been put to the test. It is, unfortunately, only applicable to the opening up of forest lands for cultivated crops and is of doubtful account in established crops where the disease is already present. This method of control is essentially one of prevention not of cure.

2—PARASITISM

(a) *Method of Experimental Infection*—*Glyricidia maculata*, a small leguminous tree grown on some tea estates in Nyasaland, has been found to be very susceptible to *Armillaria* root disease. Diseased roots of *Glyricidia* were used as a source of inoculum for experimental infection. They were cut into lengths of one foot varying from one to three inches in diameter. *Glyricidia* roots were chosen because they produced more rhizomorphs than any other host species tried in preliminary experiments.

The pieces of root were placed horizontally side by side about an inch apart and were covered by about two inches of soil. A large number of germinating tea seeds, with roots just protruding through the testa, were planted almost touching each other in the top half-inch of soil covering the *Glyricidia* roots. This method ensured that the developing tap roots of some of the seedlings would come into contact with the diseased *Glyricidia* roots, thus simulating the contact under field conditions of tea roots with infected forest roots. Other seedling roots would be likely to come into contact with any rhizomorphs developing from the infected *Glyricidia* roots.

Eighteen-months-old nursery tea plants, with stems put back to four inches and roots to twelve inches, were also transplanted in rows above horizontally placed *Glyricidia* roots, which in this case were placed fifteen inches below the soil surface. Some of the new feeder roots would thus come in contact with the diseased roots and rhizomorphs.

The soil was kept watered in both cases and the seedlings were shaded.

(b) *Technique*—The seedlings were planted in two series, the first being examined one month after planting, the others after two months. The eighteen-months-old "stumps" were examined one month after planting. The soil was washed away carefully from all roots and rhizomorphs by the use of a fine spray of water so that they were exposed as shown in fig. 1, Plate 23.

The material to be examined microscopically was fixed in formalin-acetic-alcohol. Serial microtome sections were cut so that accurate interpretation of the results could be assured. Sections which were required to show the initial stages of penetration of *Armillaria* were stained with Delafield's haematoxylin and orange G as this combination distinguished clearly the invading hyphae. Delafield's haematoxylin was used in conjunction with safranin and iodine in sections of the rhizomorph travelling in the root after penetration.

(c) *Macroscopic Observations*—The tap roots of the tea seedlings coming into contact with the diseased *Glyricidia* root frequently grew round over the surface of the latter, thereby touching the old black surface of the xylostroma exposed by the broken bark (fig. 2, Plate 23). The seedling roots in many cases grew through the bark and xylostroma and even into the soft-rotted wood of the *Glyricidia* roots (fig. 3, Plate 23). In such cases the seedling roots usually travelled horizontally in the tissues before emerging into the soil again.

Fresh rhizomorphs generally originating in isolated tufts grew into the soil from the surface of the *Glyricidia* root.

All seedling roots growing on or through the black xylostroma and in the diseased wood were healthy; *the only seedling roots to become infected were those which came into contact with the fresh rhizomorphs* (fig. 4, Plate 24).

It might be argued that these seedlings were weakened by the abnormal bend in the root, but tea roots must frequently be obliged to bend like this in the field when coming into contact with old forest roots. Many seedlings, however, were infected by the rhizomorphs growing through the soil, even though their tap roots were straight and did not come into contact with the *Glyricidia* root.

The stems of seedlings one month old are only just visible above the soil, so that there can be no question of competition for light in the seedlings planted close together in this experiment; neither can there be any starvation through overcrowding in the soil as the tap root puts out few laterals during its first month's growth. The seedlings can therefore be considered perfectly healthy during the first month of the experiment.

Only a very few seedling roots examined one month after planting showed any evidence of penetration, although quite a number had rhizomorphs attached so firmly that they were not dislodged by the spray of water used in washing away the soil. Some roots were discoloured brown on each side of the attached rhizomorph and the cortex of one or two roots was split longitudinally by the internal rhizomorph which could be seen travelling up the root as a unit.

In the two-months-old seedlings the internal rhizomorph had advanced appreciably up the root and to a lesser extent down the root for a total length of 2–12 cm., showing that the fungus had travelled actively during the second month in the seedling root.

In a few cases the rhizomorph penetrated directly from the soil into a cotyledon where it ramified through the tissues in all directions. Those rhizomorphs which became attached to the hard testa did not penetrate into the cotyledons.

In the experiment with eighteen-months-old "stumps" the new feeder roots of about one millimetre in diameter were found to have a few rhizomorphs attached.

The seedling root and new feeder roots of the "stumps" were invariably healthy except in those areas affected by *Armillaria*. A very small percentage of the infected two-months-old seedlings showed any ill-effects in the stem or leaves; in only three of them was *Armillaria* found to have advanced far enough to kill the stem completely.

(d) *Microscopic Observations*—(i) Penetration—The method of penetration appears to follow exactly on the same lines as those described so fully by Thomas. There is, therefore, no object in doing more than add further evidence in favour of his deductions. He summarizes his views as follows:

"Invasion of the root is accomplished by the penetration of a branch of the parent rhizomorph directly through the sound, healthy periderm of the host . . . The branch penetrated as a unit and was never observed to send out single hyphae into the host ahead of it. . . . Penetration is partly by mechanical means and partly by chemical means. Death of the cells always precedes the further advance of the rhizomorph into the tissue."

Figs. 5 and 6, Plate 24, show the initial stages of penetration of a tea seedling root; the photographs are clearly similar to fig. A, Plate 3, in Thomas's paper. In every case examined the rhizomorph was attached to the root by a matted mass of hyphae which filled every little indentation in the surface of the piliferous layer, no individual hyphae being observed entering the cells. No mucilaginous attachment of the rhizomorph to the root was evident. No sign of resistance in the form of phellogen was seen in any of the roots to which rhizomorphs were attached. The earliest stage of penetration is well shown up by the haematoxylin-orange G stain because the active, penetrating mass of hyphae is stained a bright purplish-blue in contrast to the light brown mass of attachment hyphae and the orange-tinted root cells.

(ii) Path of fungus in tissues of root—The invading rhizomorph advances up and down the tissues of the root, branching freely and often breaking through the cortex. The rhizomorph advances in the seedling root as a unit usually situated primarily between the cortex and xylem but sometimes in the pith only. The tip of the rhizomorph advances up the root as a massed hyphal unit. Individual hyphae were observed growing out from the sides of the rhizomorph behind the advancing tip. A number of transverse sections (fig. 7, Plate 24) of infected roots, cut approximately

5 mm. behind the tip of an internal rhizomorph, shows that in almost every case these individual hyphae grow in towards the pith (fig. 8, Plate 25) and not towards the cortex (fig. 9, Plate 25). The hyphae in the xylem and pith are fine and uniform, occupying some cells almost entirely and penetrating others with sparse branches. In a few sections hyphae were seen ramifying in the cortex in places, but these hyphae varied considerably in type, and, being usually associated with cracks in the cortex, suggested hyphae of some extraneous fungus.

Figs. 8 and 9, Plate 25, show clearly that the pith is plentifully supplied with starch while the cortex is devoid of starch. This is also the case in a healthy seedling and is not a result of parasitism.

3—INCIDENCE OF THE DISEASE

The author has found that the percentage of trees killed by *Armillaria* in virgin forest is very small, even in a pure stand of a susceptible species, yet when the forest is cut down the roots of these species become invaded by *Armillaria* to an amazing extent. This implies that once the trunk is felled the high degree of resistance of the root is broken down. The rapid spread of the fungus in this fashion is an interesting problem needing further investigation.

Under such conditions, *Armillaria* might well be judged a saprophyte or the few deaths found in the virgin forest might be caused by contact of the fungus with wounded roots. Experience in Nyasaland together with the experiments described in this paper leave no doubt that *Armillaria* is a true parasite of tea. It remains to be discovered if any environmental conditions render the tea plant resistant to attack.

Butler (1928) has shown the occurrence of *Armillaria* fructifications to be rare and the development of rhizomorphs to be sparse in Nyasaland. Infection of tea plants may be effected by rhizomorphs only or, in the opinion of the author, it may be effected by contact of the tea roots with the young frills of *xylostroma* which break through the bark of the infected forest tree roots. When young, these frills are white or cream coloured and are undoubtedly analogous to rhizomorphs. They are probably able to attach themselves to the tea rootlets and to penetrate them.

Wallace (1935) has reviewed the results of the application of certain chemicals to the soil as advocated in different countries for the control of *Armillaria* root disease. Their efficacy has not been proved over large areas of land. The old method of minimizing the risk of subsequent infection of tea on newly opened forest land by thorough stumping and root clearing has undoubtedly reduced the incidence of the disease.

Armillaria, however, is still liable to cause considerable damage. This is only to be expected as digging out forest roots to a depth of over 15 inches is almost impossible over a large acreage, and such roots have lately been found in Nyasaland infected with the fungus at a depth of four feet.

The disease could be controlled more successfully if it were possible to prevent the forest tree roots from becoming infected. It is with this aim in view that a method of biological control is suggested in this paper.

4—BIOLOGICAL CONTROL

(a)—Hypothesis

With reference to the examination of seedling infection, it seemed strange that the hyphae from the periphery of the internal rhizomorph should develop so freely in the xylem and pith and not at all in the cortex, especially in view of the general state of immaturity of a one-month-old tea seedling. Again, starch was found to be abundant in the pith and to a lesser extent in the xylem, none being found in the cortex. Combining these two observations, it was considered that *Armillaria* might require supplies of rich carbohydrates in order to grow freely in plant tissues. The author does not consider himself competent to discuss the relative distribution of the carbohydrate contents of tea seedling roots as the subject is so intricate. Priestley and Radcliffe (1924), however, found in their work on the function of the endodermis that "The primary endodermis allows the exchange of water and inorganic solutes between vascular and ground tissue but probably impedes the outward diffusion of organic solutes whilst the protoplasts are young and relatively impermeable". This tends to support the contention that the cortex of the tea seedling root is low in carbohydrate.

The xylostroma of *Armillaria* is a more massive type of vegetative growth than that of many other root parasites. So massive is it in the medullary rays that it causes longitudinal and radial cracking of large woody roots. For such a development a considerable supply of carbohydrate would seem to be necessary.

Armillaria has been shown to require media containing easily available carbohydrates for its satisfactory development in culture (Campbell, 1934).

These facts indicate the necessity of a plentiful supply of carbohydrate for the development of *Armillaria* in tree roots. Some facultative root parasites are known which can develop freely in roots low in carbohydrates, e.g., *Rhizoctonia bataticola* and *Betryodiplodia Theobromae*. From observations on the occurrence of these fungi in the roots of dead

or dying tea bushes in Nyasaland they are considered to be common saprophytes in the soil flora, only entering living roots weakened through other causes. They are clearly not virulent parasites like *Armillaria mellea*.

Storey and Leach (1933), in their work on tea yellows disease, which they showed to be due to sulphur deficiency, state that: "In 1930 Dr. W. Small drew our attention to the presence of the sclerotia of *Rhizoctonia bataticola* in the roots of bushes which had died of yellows disease. We have fully confirmed his observations. . . . We do not doubt that *R. bataticola* plays a part in accelerating the death of plants weakened by sulphur deficiency and may prevent the recovery of an extremely weakened plant. . . . The starch reserves of the root, as revealed by an iodine test of the cut end, are depleted in the diseased plant. A severely diseased plant usually contains little or no starch".

Gadd (1928) states in connexion with his views on *Diplodia* disease of tea that "Tests carried out on bushes which have died of the so-called *Diplodia* disease after pruning, have shown that in every case yet examined the roots of the diseased bush are destitute of starch. Healthy bushes, and bushes which have died of such diseases as are caused by *Poria* and *Rosellinia*, contain starch in sufficiently large quantities to be demonstrated by this test (iodine-R.L.). The absence of starch from bushes affected by *Diplodia* disease is not the result of attacks by *Botryodiplodia Theobromae* or other fungi, as this condition is to be found in live roots before they are attacked by fungi".

R. bataticola and *B. Theobromae* are, therefore, capable of developing freely in roots which have a low carbohydrate content, i.e., the exact opposite of those conditions which seem to be required by *Armillaria*.

Healthy forest trees, felled in the normal way, have their roots left with a high carbohydrate content so that they are in a suitable condition for the development of *Armillaria* if penetrated by that fungus. If some method could be employed to deplete the tree roots of starch before the trees are felled, then those roots would be rendered less susceptible to the rapid development of *Armillaria* and more susceptible to invasion by fungi of the *Rhizoctonia-Botryodiplodia* type.

The method which suggests itself most readily to bring about this condition is that of "ring barking" the trunks of forest trees near the soil some time prior to the required date of clearing the land. Ring barking entails the cutting off of a wide strip of bark down to the wood in a complete ring round the trunk. According to the acknowledged ideas of the translocation of carbohydrates in plants, this prevents the carbohydrates passing from the foliage where they are metabolized down to the roots via the phloem.

Forest areas, cleared by this method, would then be rendered free of roots invaded by *Armillaria*. The sources of danger from this fungus would thus be removed; the roots of the forest trees would be invaded instead by fungi considered to be already common in the soil and to be innocuous to plants except under abnormal conditions of growth.

(b)—Evidence in Favour of the Hypothesis

Parinarium mobola ("Muula") is a tree the roots of which are very susceptible to infection by *Armillaria* after being felled. These roots are considered to be the commonest source of *Armillaria* infection to tea on newly cleared forest land in Nyasaland. The roots tend to throw up abundant suckers after the trunk is cut down. This is probably the reason why some natives prefer to ring-bark the trunk (by fire or axe) close to the ground. Defoliation and death ensue without the production of suckers which otherwise need to be suppressed continually in the native garden.

Healthy "Muula" trees have roots containing starch as shown by the iodine test. Ringed "Muula" trees have their roots gradually depleted of starch; when such trees become defoliated as a result of ringing, their roots are devoid of starch. These roots appear to be healthy until defoliation is complete.

The hypothesis for biological control of *Armillaria* was tested by examining (a) the roots of dead "stumps" of normally felled (unringed) "Muula" trees, and (b) the roots of dead "Muula" trees killed by ringing. The former were expected to show a high percentage of roots infected with *Armillaria* while the latter were expected to show a low percentage. On carrying out a search in native gardens, dead stumps of felled trees were found to be plentiful but only a few dead ringed trees were found.

A pit, 3 feet long by 3 feet deep and 2 feet wide, was dug 6 feet away from each dead stump or trunk chosen. All roots over half an inch in thickness were examined for the easily recognizable *Armillaria*.

The dead roots not infected by *Armillaria* were all found to be dry-rotted and uniformly darkish brown in colour with an easily separable bark. These dry-rotted roots all showed a variable quantity of small black sclerotia of the *Rhizoctonia bataticola* type on the surface of the wood and imbedded in the inner bark. For reasons discussed later, these roots are referred to hereafter under the general term "dry-rotted".

The results of the examination carried out on these lines are given in Tables I and II. Six apparently dead ringed trees were found to have roots free from fungi; these trees are not included in Table II. In the

ringed trees numbered 2, 5, and 8 a few roots did not appear to have become diseased.

The Tables indicate that the ringing has made a significant difference in the percentage of roots ultimately infected by *Armillaria* and has thereby removed the main source of danger to ensuing crops susceptible

TABLE I
Roots of Dead Stumps of Unringed " Muula " Trees

Tree No.	No. of roots found		
	<i>Armillaria</i> rotted	Dry rotted	Healthy
1	12	0	0
2	9	1	0
3	10	0	0
4	7	1	0
5	6	4	0
6	0	10	0
7	13	0	0
8	8	0	0
9	9	0	0
10	0	8	0
11	10	0	0
12	6	3	0
13	9	2	0
14	12	0	0
15	8	5	0
16	10	0	0
17	11	0	0
18	7	0	0
19	8	1	0
20	0	9	0
21	11	0	0
22	0	10	0
23	12	0	0
24	8	0	0

to invasion by this fungus. The hypothesis for the biological control of *Armillaria* root disease would thus seem to be well supported.

The fungi causing dry-rot of the " Muula " roots might themselves be active parasites of tea, in which case the remedy might be worse than the disease. An experiment was carried out therefore to test this possibility. Dry-rotted roots of " Muula " were cut and planted in the same way as the *Glyricidia* roots in the *Armillaria* infection experiment described above. Some pieces of the rotted wood and bark were also used

separately. Germinated tea seeds were planted so that their roots grew down to the dry-rotted "Muula" roots. The seedlings were examined after two months' growth. Their tap roots and fine laterals were found growing in close contact with the "Muula" roots but showed no sign of being invaded by fungi. Roots of forest trees killed by ringing for the biological control of *Armillaria* therefore cannot be considered a danger to the health of a subsequent economic crop.

TABLE II
Roots of Dead Ringed "Muula" Trees

Tree No.	No. of roots found		
	<i>Armillaria</i> rotted	Dry rotted	Healthy
1	0	11	0
2	0	9	1
3	0	12	0
4	0	10	0
5	1 (?)*	8	2
6	0	11	0
7	0	6	0
8	0	10	1
9	0	8	0
10	0	10	0
11	0	7	0
12	0	11	0

* This one root was dry and desiccated, unlike the other "dry-rotted" roots; it might have been killed by *Armillaria* some years ago.

(c)—*Discussion*

The method of control of *Armillaria* root disease submitted in this paper suggests new lines of investigation. Certain large scale ringing experiments have already been started, but it will be at least three years before results are available. The author realizes that more experimental work is necessary before this method of biological control can be accepted, nevertheless, the evidence in its favour obtained to date is certainly striking. It may be of interest to other workers concerned with *Armillaria* root disease and possibly to those working on allied root parasites such as *Fomes*, *Rosellinia*, *Ustulina*, *Poria*, etc.

The essential factor in the method of biological control is the substitution of the serious root parasite, *Armillaria mellea*, by fungi incapable of causing disease to an ensuing crop via the tree roots which remain in the ground after clearing forest land. The presence of sclerotia of

Rhizoctonia bataticola in every dry-rotted "Muula" root indicated that this fungus was the primary invader of the weakened roots of ringed trees. The first tissue to be discoloured in such roots is the bark which was invariably dark orange-brown (the healthy bark being reddish), the wood being unaffected in colour. Isolations were made from the inner bark of such roots. *R. bataticola* A strain, now renamed *R. lamellifera* Small by Hopkins (1933), was only isolated twice out of twenty isolations; the other isolates have not all been diagnosed so far. There is an indication, therefore, that a number of fungi may be concerned in the biological control. These various fungi may work together in attacking the weakened roots or they may follow each other in a different order under varying soil conditions.

This work, incidentally, seems to explain Small's (1928) contention that *R. bataticola* is found in roots infected by *Armillaria*, but it does not support his statement that "when it (*R. bataticola*—R.L.) is found in the company of another fungus (or fungi) in cases of root disease it precedes the other form (or forms) in time of attack and is therefore primarily responsible for disease". *Armillaria* has been shown in this paper to be a primary parasite of tea seedlings in the absence of *R. bataticola* and to travel rapidly in the roots after penetration is effected. The infection of the bark of a tree root by *Armillaria* would have the same physiological effect as artificial ring-barking, the extremities of the root becoming starved of carbohydrates and thus losing their natural resistance to fungi of the *R. bataticola* type. The first organs to be affected in this way would almost certainly be the fine feeder roots which Small claims to be the primary seat of infection for *R. bataticola*. This would explain the presence of *R. bataticola* in roots infected by *Armillaria*.

In putting this theory of biological control into practice, it will be necessary to work out a scheduled plan for the opening up of forest areas required for the growing of crops susceptible to *Armillaria*. The roots of certain species of forest trees appear to be resistant to *Armillaria* attack after felling. In mixed forests the ringing of the susceptible species might be done many years before the land is required for cultivation, provided that the number of resistant trees left alive is sufficient to protect the land against denudation. In forests consisting of a pure stand of a susceptible species such as "Muula" (*Parinarium mobola*) it will be necessary to know the minimum time required by each species (and the different sized trees of those species) to render the roots susceptible to invasion by the innocuous fungi in place of *Armillaria*.

This time factor may be considered a disadvantage to the method of biological control. The precautionary measures lately advocated against

Armillaria root disease, however, have considerably increased the cost of clearing land for the cultivation of a susceptible crop such as tea, owing to the extra labour required for digging up roots. In comparison with this, the cost of ring-barking as advocated in the method of biological control is negligible.

ACKNOWLEDGMENTS

I am greatly indebted to Professor Sir Albert Seward and Professor F. T. Brooks, of the Botany School, Cambridge University, for their kindness in affording facilities for carrying out the microtome work connected with this paper during my leave in England. Acknowledgment is also due to Mr. E. T. Scott, also of the Botany School, Cambridge, for taking the microphotographs. I am grateful to Mr. S. T. Ashby, Director of the Imperial Mycological Institute, Kew, for naming certain fungi isolated in culture.

SUMMARY

- 1—The parasitism of *Armillaria mellea* is reviewed and discussed.
- 2—Tea seedlings were infected by the fungus under experimental conditions which are described.
- 3—Thomas's verdict on the mode of infection and parasitism of unwounded roots by *Armillaria* is corroborated.
- 4—*Armillaria* is considered to require roots with a high carbohydrate content for its free development.
- 5—*Rhizoctonia bataticola* and *Botryodiplodia Theobromae* are cited as examples of fungi, on the other hand, which are capable of developing in roots of low carbohydrate content.
- 6—The incidence of the *Armillaria* root disease and the inadequacy of the methods of control advocated up to the present are discussed.
- 7—Before clearing forest land for economic crops susceptible to *Armillaria* root disease, ring-barking the trees some time prior to felling is suggested as a biological control against the disease.
- 8—This process seriously depletes the carbohydrates in the roots, which are then invaded by relatively harmless fungi instead of by *Armillaria*.
- 9—The application to practice of this theory of the biological control of *Armillaria* is discussed.

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FIG. 1.

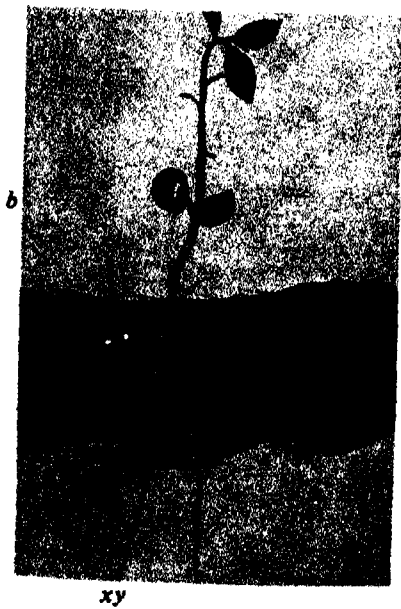


FIG. 2.



FIG. 3.

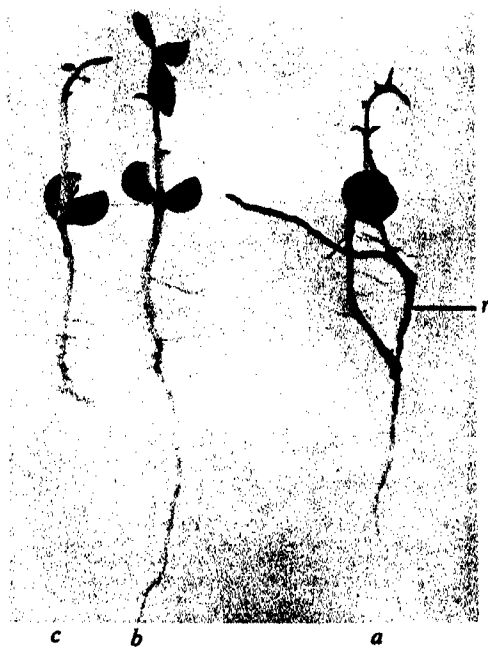


FIG. 4.



FIG. 5.

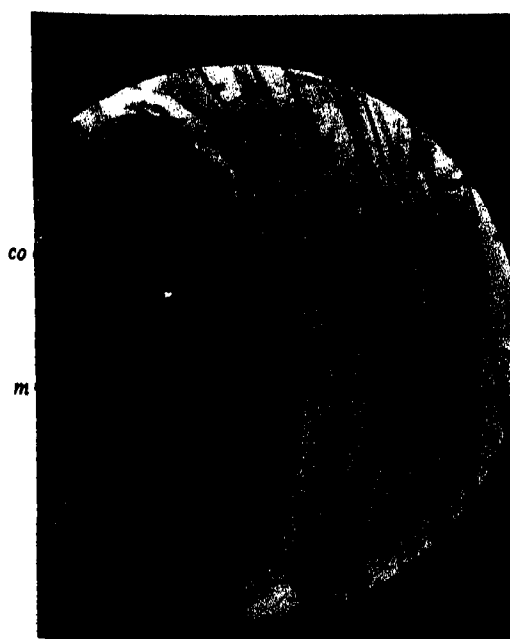


FIG. 6.

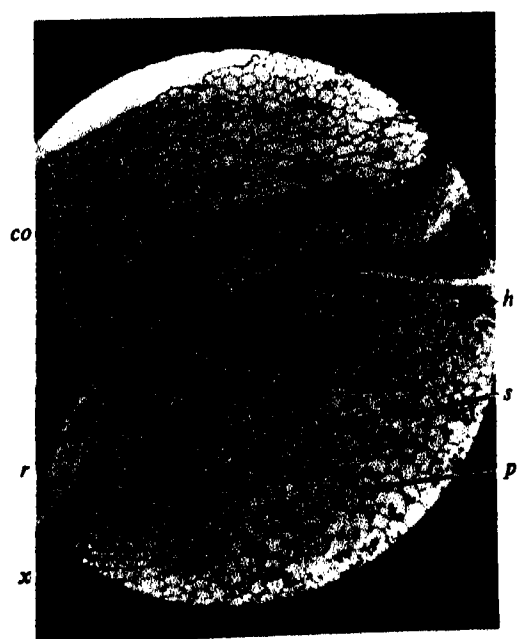


FIG. 7.

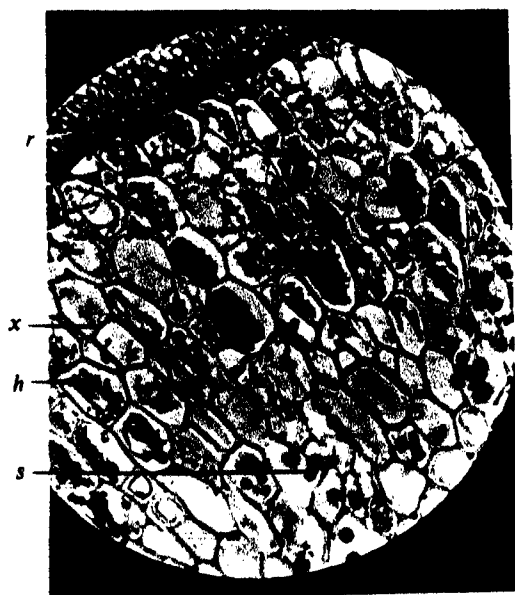


FIG. 8.



FIG. 9.

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EXPLANATION OF PLATES

Plate 23

- FIG. 1—Infection of tea seedling by *Armillaria*. Gl., infected *Glyricidia* root; *r*, rhizomorph; *c*, point of infection; *dis*, diseased seedling root; *s*, seed. $\times \frac{1}{2}$.
 FIG. 2—Tea seedling; root growing through bark (*b*) and xylostroma (*xy*) of infected *Glyricidia* root. $\times \frac{1}{2}$.
 FIG. 3—Tea seedling; root growing into wood of infected *Glyricidia* root. $\times \frac{1}{2}$.

Plate 24

- FIG. 4—(a) An infected tea seedling; *r*, rhizomorph; (b) seedling shown in fig. 2; (c) seedling shown in fig. 3. $\times \frac{1}{2}$. (Lateral roots withered by exposure, not dead.)
 FIGS. 5 and 6—L.S. of tea seedling root and rhizomorph showing initial stages of penetration by *Armillaria*. *co*, cortex; *r*, rhizomorph; *a*, attachment hyphae; *m*, massed infection hyphal unit. $\times 300$.
 FIG. 7—T.S. of infected tea seedling root. *co*, cortex; *x*, xylem; *p*, pith; *r*, rhizomorph; *h*, hyphae; *s*, starch. $\times 80$.

Plate 25

- FIG. 8—T.S. of infected tea seedling root. *r*, rhizomorph; *x*, xylem; *h*, hyphae; *s*, starch. $\times 300$.
 FIG. 9—T.S. of infected tea seedling root. *r*, rhizomorph; *co*, cortex. $\times 300$.
-

The Progesterone-like Action of Testosterone and Certain Related Compounds

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[PLATE 26]

The isolation of naturally occurring forms of oestrogenic and male hormones was soon followed by the demonstration that similar activity might be possessed, to a greater or lesser degree, by related compounds made artificially. When progesterone was isolated from the corpus luteum it was assumed that there would be an analogous lack of specificity, and investigation was immediately begun on the effect of slight changes in chemical constitution on the power to evoke progestational proliferation. In the course of this work, Butenandt and his co-workers (*see* the review by Westphal, 1935) examined a large number of compounds closely related to progesterone (pregnene-3: 20-dione), but failed to find progesterone-like activity in any. Among the more interesting of these inactive compounds were "dihydroprogesterone" (pregnen-20-ol-3-one), inactive in a 2·7 mg. dose; pregnenolone (3-ol-20-one), inactive in 25 mg.; pregnanediol, inactive in 50 mg.; pregnanediol, inactive in 50 mg.; pregnan-20-ol-3-one, inactive in 9 mg.; and androstenedione, inactive in 30 mg. With the possible exception of dihydroprogesterone, which was tested in only a small dose, this work seemed to establish the inactivity of these very closely related compounds, and by implication indicated the absolute specificity of progesterone.

A general investigation of the biological properties of compounds of the androsterone-testosterone series carried out in this laboratory during 1935-36 led to the examination of certain of the compounds, especially those methylated or ethylated in position 17, for progesterone-like activity (*see* Ruzicka, 1936, for discussion of the chemical relationships). To our surprise, in view of Butenandt's results, certain of these compounds proved to be active (Klein and Parkes, 1936), and fuller investigation has shown that seven of them exert the specific activity previously thought to be restricted to progesterone.

* Rockefeller Travelling Fellow.

TECHNIQUE

The routine test was carried out as described by McPhail (1934). Immature rabbits, 500–800 gm. body weight, were given 5 γ of oestrone on alternate days for 6 days, and the substance under test was given over 5 days following. In exceptional tests, (a) small rabbits 200–300 gm. were used, (b) the ovaries were removed, and (c) methyltestosterone was tested by the Corner-Allen method on the adult rabbit ovariectomized immediately after mating. Positive responses were graded +, ++, +++, and +++++ (full proliferation) as described by McPhail.

The compounds were dissolved in arachis oil, and injected subcutaneously. At the time these experiments were being carried out, it was demonstrated by Miescher, Wettstein, and Tschopp (1936) that the activity of testosterone could be much increased by adding fatty acids to the oil solution, a result almost certainly brought about by retardation of absorption. In certain cases, therefore, a total of 1 gm. palmitic acid was added to the solution of male hormone given to the rabbits.

EXPERIMENTAL RESULTS

Non-Methylated Compounds—Of six non-methylated compounds (Table I), only testosterone and androstenedione showed definite signs of progesterone-like activity. Androsterone (10 mg., 25 mg.), androstanediol (20 mg.), *trans*-dehydroandrosterone (10 mg.), and *trans*-androstenediol (10 mg., 50 mg.) evoked no response. Testosterone, however, as shown in Table I, produced a positive response in a dose of 20 mg., or in one of 10 mg. if given with palmitic acid (fig. 4, Plate 26), while androstenedione, inactive in 10 mg. and 30 mg. in the normal test, yet evoked some progestational response in a 200–300 gm. rabbit when a dose of 50 mg. was given. The difference from Butenandt's result with this substance is easily accounted for by the smaller size of the test rabbit and the higher dosage.

Methylated Compounds—The addition of a 17-methyl group obviously increases the similarity to progesterone of the derivatives of androstane and androstene. We have been able to examine the effect of this addition on the biological activity of three compounds—androstanediol, testosterone, and *trans*-androstenediol. Methylation in position 17 increases the progesterone-like activity of testosterone (fig. 3, Plate 26) and confers activity on androstanediol, but in the one experiment we were able to perform with methyl-*trans*-androstenediol, 10 mg. showed only the

slightest signs of activity. A fourth methylated compound, methyl-dihydrotestosterone, was found to be active, but as the corresponding non-methylated compound was not available, it is not possible to say how far this activity is due to the methylation.

TABLE I

Compound	Total dose (mg.)	Proliferation index	
		Without palmitic acid	With palmitic acid
<i>Saturated compounds—</i>			
Methylandrostanediol (Cis)	10	++	
	10	+ to ++	++
	15		+ to ++
	20	+ to ++	
Ethylandrostanediol (Cis)	10	+ to ++	++ to +++
	20	? slight	++ to +++
Methyldihydrotestosterone	7½	+ to ++	
Ethyldihydrotestosterone	10		++
	20	+++	+++
<i>Unsaturated compounds—</i>			
Androstenedione	10	0	
	30	? slight	? slight
	50	+	
Testosterone	10	? slight	+ to ++
	10	? slight	++
	20	+ to ++	
Methyltestosterone	7	+ to ++	
	10	++	+++
	10	+ to ++	+++
	10	++	
	20	+ to ++	

Ethylated Compounds—In two compounds it is possible to compare the effects of methylation and ethylation in position 17. Ethylandrostanediol appears to have much the same activity (fig. 5, Plate 26) as the methyl compound, and the same applies to ethyldihydrotestosterone (fig. 6, Plate 26) so far as the very inadequate data go. *Trans*-androstenediol remains apparently inactive after ethylation as it does after methylation, doses of .5, 10, or 20 mg. of the 17-ethyl-derivative evoking no response.

Addition of Palmitic Acid to the Solution Injected—Retardation of subcutaneous absorption by addition of palmitic acid to the solution injected appears to increase the effectiveness of ethylandrostanediol, testosterone, and methyltestosterone. The same tendency is not evident with ethyldihydrotestosterone or methylandrostanediol, but the data are very meagre. On the whole, it seems justifiable to say that while some increase in effectiveness results from the addition of palmitic acid the difference is not nearly so great as that in the male hormone action of testosterone on the rat. It may be assumed tentatively that the wastage of injected free hormone by destruction and excretion is less in the rabbit than in the rat. This supposition is in keeping with an experiment in which 10 mg. of testosterone acetate was found to give little better response than 10 mg. of the free hormone. There was no sign of the great increase of effectiveness shown by the esterified form on the castrated rat.

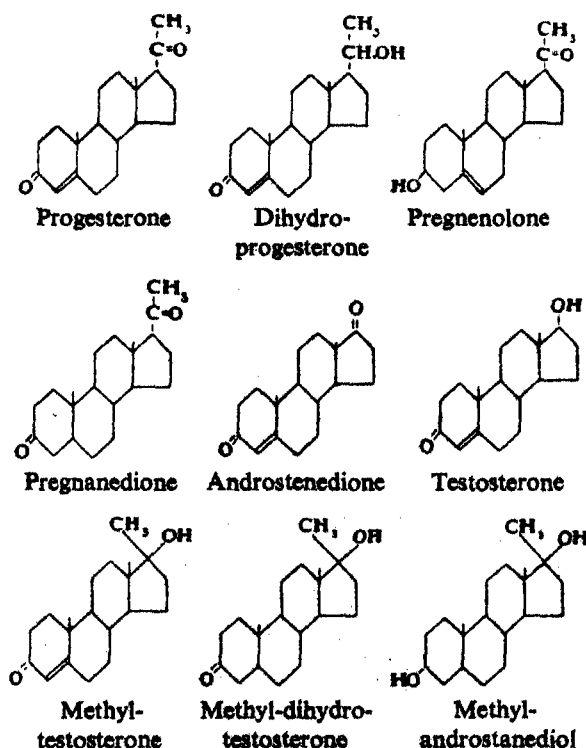
Removal of the Ovaries—The unexpected nature of the results recorded above made it necessary to consider the possibility of the progestational proliferation of the uterus being due to stimulation of the ovary, either directly, or indirectly through the pituitary. Two experiments were performed. In the first, two adult rabbits were mated and immediately ovariectomized. One was given five daily does of 2 mg. methyltestosterone and the other kept as a control. Both were killed 5 days after ovariectomy. The one receiving the methyltestosterone showed a +++ progestational proliferation, the control no proliferation of any kind (figs. 1 and 2, Plate 26). In the second experiment, four immature rabbits were given the usual preliminary oestrone treatment, followed by 10 mg. testosterone plus palmitic acid. The ovaries were removed from two at the beginning of the experiment. At the end of treatment the ovariectomized animals both showed a + to ++ proliferation. The two with intact ovaries showed slightly less.

These experiments demonstrate conclusively that the progesterone-like activity of the compounds is not due to direct or indirect stimulation of the ovary or to conversion of the compounds into progesterone by the ovary.

DISCUSSION

Owing to the large amount of material required, no attempt was made to do regular group tests on the compounds, but, so far as it is possible to judge from the qualitative data recorded above, methyl- and ethylandrostanediol, methyltestosterone and methyl- and ethyl-dihydrotestosterone all seem to have about the same degree of progesterone-like

activity. If one assumes that 10 mg. of these will produce a ++ proliferation, they are about one-twentieth as active as progesterone (quite a high degree of activity compared with that which is considered good in the case of artificial oestrogenic compounds compared with oestrone). Testosterone is less active, and androstenedione less again. Amounts sufficient to give a full ++++ proliferation have not been given, so that there is no decisive evidence of the capacity of the male hormone compounds to produce a full proliferation. There would seem to be no doubt, however, that the proliferation produced is similar to that resulting from progesterone treatment.



Graphic formulae of the chief compounds referred to

Differences in technique, sensitivity of animals, etc., make it impossible to compare the results quantitatively with those of Butenandt on pregnenolone, etc., but, qualitatively, the results definitely disprove the supposed specificity of progesterone. Neither the diketone character nor the double bond in ring I is essential; even the short side-chain can be dispensed with. In view of this, it seems extraordinary that pregnenolone should not have shown activity. It must be pointed out, however, that



FIG. 1.



FIG. 2.



FIG. 6.



FIG. 5.



FIG. 3.



FIG. 4.

(Facing p. 578)

androstenedione, because of its diketone character, is more similar to progesterone than is testosterone, but it has less progesterone-like activity.

The various compounds used in the experiments described above were generously made available by Professor L. Ruzicka, Dr. K. Miescher, and Messrs. Ciba, to whom our best thanks are due.

We are also grateful to Professor A. Butenandt and Dr. R. K. Callow for criticism, suggestions, and information.

SUMMARY

Methyltestosterone, methyl- and ethyl-dihydrotestosterone and methyl- and ethyl-androstanediol all show appreciable power to evoke progestational proliferation in the rabbit uterus. They are about *one-twentieth* as active as progesterone. Testosterone shows some activity and androstenedione slight.

The anomalous activity of these male hormone compounds is exerted in the absence of the ovary, and is therefore not due to direct or indirect stimulation of it, or to conversion of the compound to progesterone in the ovary.

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 Westphal (1935). 'Ergebn. Physiol.,' vol. 37, p. 273. This paper gives a full résumé of the results obtained by Professor Butenandt and his colleagues.

DESCRIPTION OF PLATE 26

- FIG. 1—Uterus of adult rabbit ovariectomized after mating and killed 5 days later. No progestational proliferation.
 FIG. 2—Uterus of adult rabbit ovariectomized after mating and killed after 5 days' treatment with 2 mg. daily methyltestosterone. +++ progestational proliferation.
 FIG. 3—Uterus of immature rabbit sensitized with oestrone and then given 10 mg. methyltestosterone plus 1 gm. palmitic acid over 5 days. +++ progestational proliferation.
 FIG. 4—Uterus of immature rabbit sensitized with oestrone and then given 10 mg. testosterone plus 1 gm. palmitic acid over 5 days. + to +++ progestational proliferation.
 FIG. 5—Uterus of immature rabbit sensitized with oestrone and given 10 mg. ethyl-androstanediol plus 1 gm. palmitic acid over 5 days. ++ to ++++ progestational proliferation.
 FIG. 6—Uterus of immature rabbit sensitized with oestrone and given 20 mg. ethyl-dihydrotestosterone over 5 days. +++ progestational proliferation.
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Discussion on the Chemical and Physical Basis of Pharmacological Action, 12 November, 1936

Opening Address

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This subject is of basic importance in pharmacology and in chemotherapy and is becoming of increasing importance in physiology and in enzyme chemistry. Hence any quantitative information that can be obtained is of great value. These reasons justify the attempt to apply methods of physical chemistry to living cells, even though the latter are very unsuitable material for such work. It must, however, be remembered that such an attempt implies using methods for purposes for which they are not intended, and this is always a dangerous procedure.

The living cell is so complex and its mode of organization and structure so imperfectly known that the number of uncontrolled and even unknown variables is always great, whilst unavoidable sources of error such as individual variation prevent any high degree of accuracy being attained. Only the simplest methods of mathematical analysis are appropriate in such conditions, and formal proof of any hypothesis can rarely be obtained. We must therefore be content with finding explanations for drug action which are reasonably probable and involve the fewest unproven assumptions.

The logical approach to the subject is to determine quantitatively the amounts of drugs reacting with cells, and then to determine the kinetics of drug action. Quantitative measurements will show the maximum quantity of a drug that can be involved in a pharmacological action, but in most cases such actions are probably produced by only a small fraction of the total quantity of drug fixed.

The study of enzyme poisons shows that with purified enzymes the amount of silver reacting with the active group may be only 10% of the quantity fixed (Sumner and Myrbäck, 1930) and for silver acting on intracellular enzymes this figure falls to about 0.5% (Euler and Wallis, 1924).

Quantitative data show that selective adsorption is an important feature of drug action. The oligodynamic action of metals on algae (lethal action in a concentration of 1 in 10^6) was mysterious until it was shown that the actual concentration of metal in the algae was several parts per

10,000 (Eichholtz, 1934). Many pharmacological problems are simplified if instead of comparing the drug action with the concentration of drug in the solution, we consider the probable concentration of the drug at the cell surface.

KINETICS OF DRUG ACTION

The penetration of dyes into the internal vacuoles of *Nitella* and *Valonia* (Osterhout, 1933) follows the monomolecular formula, and this suggests a simple diffusion process. The internal concentration may, however, be ten times as great as the external concentration, and therefore the process must be much more complex than is indicated by a study of its kinetics.

The rate of production of a biological effect gives little information regarding the rate of chemical action, unless biological lag can be excluded. In many cases there is a long interval between drug fixation and the appearance of biological response. In certain favourable cases drugs appear to combine with surface receptors and produce an immediate biological response, but these effects usually are produced too quickly to permit accurate time measurements.

In other cases the process of drug uptake appears to be one of sorption. A rapid adsorption process is followed by a slow diffusion into the interior of the cell. For example, mercury perchloride is fixed in a few minutes by bacterial spores but may take days to produce death. For these reasons the measurement of the rate of appearance of a biological response gives very uncertain information about the rate of reaction between the drug and the cell.

When drugs destroy populations of small organisms (bacteria, red blood corpuscles, yeast cells, algae, etc.), the death rate is frequently constant. (Linear relation between log survivors and time.) Constant death rates are sometimes found with larger organisms (*Drosophila*, mice, etc., Gowen, 1934). The occurrence of this relation has been regarded as a proof that a monomolecular reaction occurs between the drug and the organism (Arrhenius, 1915; Chick, 1930; Rahn, 1934). This implies that life depends on some vital molecule and that the chance of drug reacting with the vital molecule of any single organism remains constant throughout the exposure to the drug. Quantitative evidence shows that when a drug such as phenol acts on cocci, each coccus fixes 10^5 to 10^7 molecules (Liese and Mendel, 1923). The fixation is a complex sorption process.

The monomolecular theory of drug action also assumes that individual variation is negligible, although all evidence agrees in showing that

extensive individual variation in response to drugs occurs with all cell populations.

If cells vary individually in respect of the amount of drug that must penetrate in order to produce death, and the penetration follows a diffusion process, then the individual variation in respect of time until death will have a skew distribution. This seems much the simplest method of explaining the occurrence of these constant death rates (Clark, 1933, *d*).

The quantum theory of cell destruction by radiation is a parallel hypothesis to the monomolecular theory of drug action. Quantitative estimations have shown that millions of quanta are absorbed per cell before death is produced and hence this theory also has necessitated postulating a sensitive spot or vital centre in cells. In this case also the results can be explained as an effect due to individual variation (Clark, 1933, *e*; Pugsley, Oddie, and Eddy, 1935).

CONCENTRATION-ACTION RELATIONS

Actions produced by drugs can be divided into two classes: graded actions which may be proportional to the amount of drug fixed by the cell, and all-or-none effects which occur when the amount of drug fixed reaches a certain level. The latter class gives less information than the former regarding the relation between chemical action and biological response. The action of drugs on proteins which carry an active group appears to be a favourable line of approach to the study of drug action on cells. Enzyme poisons show at least three types of relation between drug concentration and amount of action. (a) Hyperbola, (b) linear, (c) sigmoid (Rona *et al.*, 1921, 1922). If a drug is present in considerable excess and there is a reversible reaction in which a receptor combines with one molecule of the drug, then the relation between drug concentration (x) and response (y) expressed as per cent of maximum follows the formula ($Kx = y/(100 - y)$). This formula also expresses the simplest forms of adsorption (Langmuir). Many concentration-action relations of enzyme poisons approximate to this formula.

With narcotics a linear concentration-action relation is obtained. Sigmoid concentration-action relations are occasionally obtained with enzyme poisons and can be explained as all-or-none effects produced on the protein carrier.

The concentration-action curves obtained with oxygen, or carbon monoxide, and haemoglobin, show that a relation, which under the simplest conditions follows the formula given above, may be distorted when the conditions become more complex. Similarly in many cases

where the relation between concentration of enzyme poison and inhibition of purified enzyme is a hyperbola, this relation is distorted when the enzyme poison acts on intracellular enzymes (AgNO_3 , Euler and Walles, 1924; quinine, Rona and Nicolai, 1927). The three types of concentration-action curves mentioned above were found in pharmacological experiments by Storm van Leeuwen (1923). They can all be obtained on a single tissue such as the frog's heart (Clark, 1933, *c*) *e.g.*,

Hyperbola—acetylcholine, cyanide

Linear relations—narcotics

Sigmoid curve—potassium chloride.

A single drug tends to show similar concentration-action relations with different tissues, *e.g.*, narcotics, cyanides, KCl.

The writer explains these curves as follows. The sigmoid curves are due to the drug producing some all-or-none effect on the cells. The linear relation obtained with narcotics can be explained as a portion of an adsorption curve. The hyperbolae indicates the formation of a reversible compound with receptors on the cell surfaces.

THE MODE OF ACTION OF ACETYLCHOLINE

This drug is very important and has been studied extensively, hence it is a good example to consider. The following information exists. An action can be produced on an isolated tissue in about one second. Evidence from nerve stimulation suggests that an action may be produced in a few thousandths of a second. Methylene blue certainly acts on the heart surface and it antagonizes acetylcholine (Cook, 1926). These facts indicate that the drug acts on the heart surface. This view accords with the fact that sensitive tissues contain large quantities of acetylcholine. Presumably the drug when inside the cell has no action. Micro-injection experiments have proved this to occur with narcotics and cyanides (Clark, 1933, *b*).

Quantitative experiments show that about 10,000 molecules of acetylcholine may produce an effect on a heart cell. The quantity would only cover $1/6000$ of the surface (Clark, 1933, *a*). This suggests that it acts on special receptors. Acetylcholine is a strong base and therefore the receptors are probably carboxyl groups. The combination is presumably dependent on some special "pattern" associated with the carboxyl group, which fits the pattern of the acetylcholine molecule. Antagonists such as atropine, methylene blue, etc., presumably act by altering the pattern of the receptor.

The factors which increase tolerance to acetylcholine are only partially understood, and the potential theory of drug action is based on unexplained effects of this character. The writer believes that the chemical theory of drug action outlined above is preferable to the potential theory of drug action because the former explains more of the known facts and does not involve the assumption of mechanisms unknown to physical chemistry.

CHARACTERISTIC CURVES

These are curves relating concentration or dose of drug with the incidence in a population of some all-or-none effect. The sigmoid concentration-action curves are assumed to be characteristic curves.

With many drugs characteristic curves are of symmetrical sigmoid shape, and can be explained as expressions of individual variation distributed in a symmetrical manner. With some drugs highly skewed characteristic curves are obtained, which become symmetrical when plotted against log dose. Some of these curves indicate an enormous range of individual variation. There is an obvious correlation between the shape of concentration-action curves and characteristic curves. The skew characteristic curves are found where the concentration-action curve is a hyperbola. It is difficult to find any simple explanation for this correlation.

W. STRAUB (München)—*Mode of Action of Stimulating Alkaloids*—Alkaloids usually have a specific narcotic action which they exert according to the law of narcosis. The specificity is still a problem. We know that in the organ specifically influenced a process of loading takes place (Straub, 1903, *a, b*) which results in a high concentration of alkaloid inside the cell; this process is reversible like the dyeing of a fibre. The degree of action is proportional to the amount stored.

Some alkaloids, like muscarine, pilocarpine, and others, have a *stimulating effect* similar to that of vagus stimulation.

These alkaloids, too, are stored reversibly inside the cell at a high concentration (Straub, 1905, 1907), but the relation between the action and the storing process is more complicated than that of the narcotic alkaloids. The action, *i.e.*, the inhibition of the heart in the case of muscarine, is exerted only *during* the process of loading and ceases as soon as this process has reached its final equilibrium or is forcibly interrupted, fig. 1. In a system of an isolated heart, plus saline, the effect takes place with the speed of an ionic reaction or an electrical vagus stimulation; it passes away slowly but completely without external interference. If the poison

is removed from the system during the height of the effect, recovery occurs almost immediately. The quantitative estimation of the poison distributed between the heart and the saline shows that in the final stage of recovery all the poison is contained inside the heart muscle whereas the solution outside is practically free from poison.

Thus the *process of penetration* into the cell is decisive for the action of the poison and the effect is the more intense the steeper the concentration potential between outside and inside. Since the concentration of freely dissolved poison inside is almost zero by adsorption, there will be an active concentration potential as long as there is any poison at all outside. For this reason such poisons have been called potential poisons.

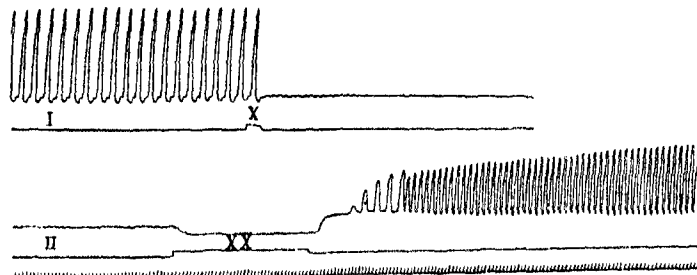


FIG. 1—Straub (1905). Isolated heart and saline. At *x* muscarine solution was added, at *xx* it was removed without washing out. Distribution: at *xx* active quantities of muscarine are present in the heart muscle and in the solution. II is the immediate continuation of I.

The place of action on which the poison exerts its effect must be a peripheral cell organ, which during the passage of the poison is deformed to such a degree that the function of the cell ceases. It is probable that this peripheral organ is the cell membrane or something which is functionally similar.

On the heart and on the skeletal muscle the application of a muscarine solution at one point causes a deformation of the membrane, which is followed by a monophasic action current or a maximal demarcation current respectively, exactly like that produced by local application of a potassium salt or cauterization (Straub, 1912; Evans, 1912).

The hypothesis of the potential action of poisons similar to muscarine which originally was a pharmacological conception, becomes interesting from the biological point of view since acetylcholine which chemically is similar to muscarine has been recognized as a substance naturally occurring in the body, namely the vagus substance, the humoral transmitter of vagus stimulation.

The action of acetylcholine is also determined by potential conditions (Straub, 1936). It can cause also the electrical membrane phenomena obtainable on the heart and on skeletal muscle described above (Straub, unpublished).

The biological peculiarity of the action of acetylcholine is the fact that by intracellular saponification due to an esterase the concentration in the cell is at a continuous minimum which is probably zero, therefore the equalization of potential always occurs at maximal steepness and with maximal effect. Because of these special conditions it must be possible to maintain an effect of unlimited duration with acetylcholine and not only to produce a short stimulus. In this interplay of potential conditions with the action of the esterase constantly preventing an equilibrium, we

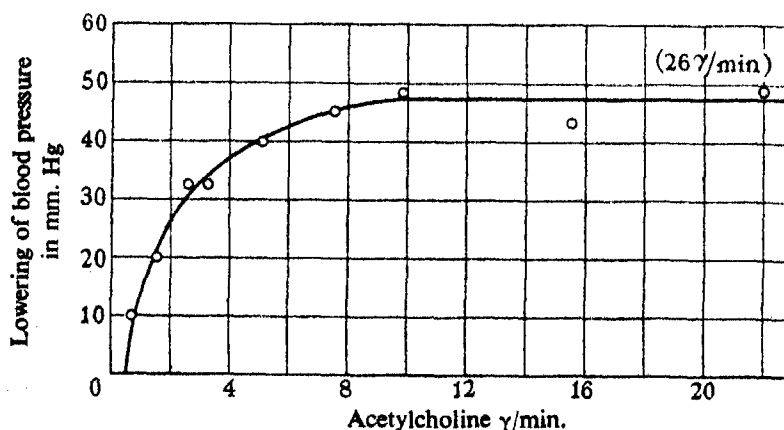


FIG. 2—Gremels and Zinnitz (1935). The characteristic for the action of acetylcholine on the blood pressure of the cat.

have the explanation why acetylcholine is fit to be a chemical regulator and thus a hormone of the organism.

If, for example, the function of acetylcholine is to keep the blood pressure constantly lowered to a certain extent, this can only be obtained by a dynamic equilibrium between the supply (secretion) of acetylcholine and the intracellular destruction by the esterase.

The blood pressure of a cat can be lowered by 50% by a single injection of 0.1 γ of acetylcholine, but only for a very short time; if the low blood pressure is to be maintained constantly, it is necessary to infuse acetylcholine, copying a process of secretion, at a rate of 5.1 γ per minute. Not the absolute amount, but the concentration is important.

If for all obtainable lowerings of blood pressure the necessary amount of acetylcholine per minute is estimated, the figures are found to lie on an exponential curve, which can be called the characteristic for this

hypothetical hormone action of acetylcholine, fig. 2. The curve does not start at zero, but at 0.05γ per minute; it reaches the maximum at 10γ per minute and after is horizontal (Gremels and Zinnitz, 1935).

It is not yet clear whether acetylcholine is a hormone which is used to exert a distant effect or whether it is only the active transmitter of vagus stimulation locally. On the other hand, the hormonal property of adrenaline, the sympathetic substance, is beyond doubt; it is constantly liberated by a gland into the blood stream and can mobilize sugar as long as it is liberated. That the action of adrenaline depends on potential conditions has been known for some time (Straub, 1907; Straub and Kretschmer, 1907); the maintenance of a concentration gradient between the outside and the inside of the cell is due to the fact that it is very easily oxidized. For this reason adrenaline is analogous to acetylcholine. The characteristic curve of adrenaline is exactly the same as that for acetylcholine; if infused intravenously, each rate of infusion (secretion) expressed in mg. per minute corresponds to a certain increase of blood pressure. Neither does this curve begin at zero, there is a rate of secretion which produces no effect, a kind of chemical adrenaline tone.

It is quite probable that adrenaline and acetylcholine are not the only substances naturally occurring in the body, the action of which depend on potential conditions. There may be other hormones, the absolute absorption of which into the cells never reaches an equilibrium and the effects of which, therefore, are dependent on the concentration in which they are secreted into the blood.

R. A. PETERS, F.R.S.—*Biochemical Conception of Cell Organization*—Some conception of the nature of the organization of chemical compounds in the living cell is essential in an attempt to place the action of drugs upon cells upon a rational rather than an empirical basis. The older biochemical concept of the cell as a comparatively unorganized colloidal solution has had much value, especially in its success in rescuing the chemistry of the cell from the belief that all living activities necessarily involved large and therefore experimentally inaccessible molecules (Hopkins, 1912). Between the physico-chemical and the stereo-chemical the boundary has become less well defined of late owing to the development of surface chemistry; but taking the broad view, there are changes in cell activity which can be quite well explained upon the hypothesis that changes in colloidal aggregation at the surface of a cell will induce alterations in cell activity. Apart from the well-known phenomena connected with fertilization and ciliary movement, I can quote two less well-known instances. J. S. Young (1930) found that active proliferation in

the epithelial cells of the rabbit lung can be produced by the injection of electrolytes into the pleural cavity, and that the effect follows the valency of the cation, as for colloids. Glycolysis in slices of brain tissue can be controlled to some extent by changes in the proportions of neutral salts (Ashford and Dixon, 1935; Dickens and Greville, 1935). But the temptation to simplify the problem of drug action upon lines such as these breaks down upon a deeper inspection of other phenomena.

In particular, a consideration of the problems raised by the pharmacological action of the toxic chemical compounds used in warfare will soon lead to the conclusion that no such simple idea of "colloid interference" will explain their actions. Even if we exclude the complicating effects of the highly organized physiology of the higher animals and investigate the action of arsenical compounds upon cultures of protozoa, we find that slight changes in chemical structure make enormous differences in toxicity, a feature which may be regarded as a comparatively unspecific one to investigate. Walker (1928), in a rather detailed investigation in my laboratory of toxicity to *Colpidium*, found that among trivalent arsenic compounds, the primary has only 1/20-1/100 of the action of secondary compounds, and among the latter comparatively slight structural changes made a considerable difference in activity. To fall back upon solubility and penetration in explanation of these results gives no mental satisfaction. The idea that the cell is a comparatively unorganized colloidal solution provides no foothold for a possible hypothesis here, nor does it help with the fundamental problem as to how the living matter maintains its general organized character.

As the result of several years' reflexion upon these and related problems, in 1929-30 I advanced the postulate that cells possessed what Needham (1936) has admirably called a "cytoskeleton". This really means a confession of failure to think adequately of molecular arrangements in the complete cell without concepts of space (such as are used in organic chemistry) as well as of time. The conception of such an organization follows readily from the development of surface chemistry upon the lines initiated by Hardy, Langmuir, and Harkins. The suggestion is that the cell is organized so that there is in effect a three-dimensional mosaic extending throughout the cell consisting of a network of protein molecules. As a corollary, the cell surface proteins will be connected to the directive proteins of the cell nucleus by threads of cytoplasmic protein; the enzymes of protein nature would naturally be closely associated with this structure and so in large part be under the influence of activity of the mosaic. Such a sub-microscopic structure would be the cell counterpart of the nervous system in the higher animal. It might

transmit the equivalent of impulses either by actual mechanical change, alteration in orientation of its constituents, or by alterations of electronic balance. It must be noted that if the idea of space organization in cells proves a useful hypothesis, it is surely legitimate to postulate it. In pure organic chemistry, recognition of the possibility that 6 carbon atoms formed an organic structure in benzene, with all its implications, has been no less useful because there may be still difficulties in deciding the exact configuration of the benzene nucleus in electronic terms.

An orientation of the mind in this direction soon leads to interesting developments. That proteins as such can initiate morphological change in cells was demonstrated in particularly clear form in the classical experiments of Dakin and Dale (1919), upon the correlation between the anaphylactic reactions of albumens and differences in organic chemical structure of the proteins. By following what is already accepted about the proteins of the nucleus, taking the protamine constituent, and making use of the gene theory, I pointed out (so far as I know for the first time*) that the significant groups in the direction of this chemical change are likely to be the —NH_2 , —COOH , guanidine from arginine, glyoxaline from histidine and —CONH— , the particular pattern upon which they are constructed influencing the final results. The role of the nucleic acid of which much is often made would appear to be secondary, and also of the polar groups such as —OH in their relation to hydration. Water is of course essential to life, but I do not think that there is much evidence that rapid local changes in hydration are an essential ingredient of all living activity. The problem therefore simplifies to the activity of the groups mentioned in their various possible relations.

A particularly important feature of the view adumbrated is that it makes it possible to understand in chemical terms how independent chemical reactions could run in various parts of a cell while at the same time the stimulus applied to the surface of the cell could be transmitted as a whole throughout the cell by the three-dimensional mosaic, just as in a nervous system.

Since my original lectures, the way has been smoothed in some respects for the "cytoskeletal" hypothesis; there is much evidence, from X-ray work especially, that proteins may exist as long rod-shaped structures (Meyer and Mark, Astbury, Bernal and colleagues) which could be conceived as having a semi-rigidity under the right conditions, though this is not characteristic of all proteins in solutions as Svedberg has shown. As part of the magnificent work upon these lines proceeding in the Carls-

* These points have been recently developed by Wrinch (1936).

berg Laboratory (Linderstrøm, Lang, and Rasmussen, 1935), clupein has been found upon titration to behave as a simple unbranched polypeptide chain with one terminal —COOH and one terminal N group, the content of amino acids being 19–20 arginine residues and 7–8 mono amino acids. Proteins also will spread to thin films at an interface, and the protein models suggested by Lloyd and Wrinch (1936) provide details for such organization; according to Danielli and Harvey (1935), proteins together with lipoids exist at the surface of mackerel eggs. In regard to any more direct proof of the presence of a permanent organized structure in the cytoplasm, the situation is not much better. Moore (1936) from *Plasmodium* experiments believes that a sub-microscopic structure must exist, and Seifriz (1928)* that a fibrillar structure is the best interpretation of the elastic properties of protoplasm; but accepting such fibrillar units, it seems that the nearest we can get to structural permanency in cytoplasm is that of the thixotropic gel. I do not consider this to be an insuperable objection to the cytoskeletal hypothesis.

I have dwelt in some detail upon these views because I wanted to make it clear that there was a sufficient basis in what is known about the chemical constitution of the cell for their advance, and because I think that if they contain a germ of truth it must alter our mode of approach to some pharmacological problems. I am not blind to the possibility that a measure of organization can be reached by the successive action of selective enzymes. The poisoning of one of such stages by a drug would be enough to check an energy yielding process, and if the cell could not substitute some other one, to cause death. Even here, however, as Needham points out, organization is often implied. There are the experiments of Case (1931), indicating some structural organization of glycolytic enzymes; Thompson and I can quote another from our own experience; the lactate and pyruvate oxidase systems in brain tissue seem to be structurally related. Dr. J. C. Eccles informs me that he has experiments requiring for interpretation the idea that response can be propagated through the nerve cell body as well as over its surface. I think that if we look for such evidence with our eyes open, we shall find several other examples which point to similar evidence of structure within the cell.

If in the main the surface protein (S.P.) is considered to be the acceptor, (receptor in Professor Clark's sense†) and the cytoplasmic protein (C.P.)

* A view of the structure of protein unknown to me at the time, but much like my own.

† There would be room upon the surface of one *B. coli* for some 50 million receptors of the size —COOH .

the conductor, we can predict what is the nature of accepting groups, either the active groups of proteins themselves, the prosthetic groups of enzymes which are associated with proteins, or of groups belonging to molecules in very intimate contact with proteins.

The unspecific colloidal reagents must interfere and cause cellular disturbance because proteins are susceptible to the ordinary reactions of negatively charged colloids. The more specific reagents will act upon specialized proteins; this would make the special receptors mentioned by the opener groups belonging to proteins. In regard to the point that narcotics act upon the surface and not when in the interior of a cell, we should expect this if the interior is conducting in character. Like the brain, it would be relatively insensitive. The growth of tolerance introduces a complication of great difficulty upon any hypothesis, but I believe that the kind of view put forward here is more hopeful than others. We must assume in the first instance for simplicity that the essential nature of the S.P. remains unchanged, but if this is under control of the mosaic, partial rearrangements may be brought about by external stimulus.

There has been one possible difficulty from the first in considering that some of the ionizable groups in proteins of special type could be responsible for the direction of cell activity, of which I think that I have largely disposed. At the p_{H_1} of the cell (about neutral) simple $-\text{COOH}$ and $-\text{NH}_2$ groups would be completely dissociated and present entirely in salt form if in simple solution p_K approx. 4.8 and 10.0. I have been able (1931) to show that $-\text{COOH}$ and $-\text{NH}_2$ groups in an interface oil-water have their apparent dissociation constant altered, so that the region over which change in ionization occurs is that of the cell contents.

In conclusion, when we deal with the common ground between the pharmacologist and the biochemist at the level of research upon the cell, the most hopeful line for each seems to be the attempt to decide how many various types of compounds will interfere in cell activity, and to determine the exact method of this interference, or the possibilities which can be reconciled with the known structures. This is equivalent to finding the number of possible receptors, and hence the great value of such work as Professor Clark described upon drug antagonisms. In some cases there appears to be already a most hopeful future. It seems likely that not only the fundamental action of arsenic compounds and that of iodoacetic acid upon the cell may be solved by knowledge of their intimate action with either $-\text{SH}$ or in the latter case perhaps also nitrogen groups; but that a solution of the problem of mustard gas action may be

reached through knowledge of the action of iodoacetic acid,* with which its effect upon pyruvate oxidase is so similar that in all probability similar receptors are involved.

J. H. QUASTEL—*Effects of Narcotics on Tissue Metabolism*—Earlier work (Quastel and Wheatley, 1932, *b*) has shown that narcotics at low concentrations have the property of greatly inhibiting the respiration of brain tissue. It was established that the substances whose oxidations were most depressed by the presence of narcotics were glucose and its breakdown products, lactic and pyruvic acids. Substances such as glutamic acid and succinic acid, which are also burned by the brain, were not so greatly affected by the narcotics—in fact, the oxidation of succinic acid was not at all inhibited at the low concentration of narcotics investigated. The narcotics were found not to interfere with the access or activating power of oxygen; their effects were chiefly restricted to the systems concerned with glucose, lactic acid, or pyruvic acid. Another fact established was that among narcotics of the same chemical type those with the greatest narcotic power had also the greatest inhibitive power. The hypothesis advanced was that narcotics may exert their physiological effects by inhibiting the oxidation of glucose (which is the most important source of energy to the brain) at the nervous structure at which the narcotic is adsorbed. This work was carried out on the minced brain tissues of rat, guinea-pig, rabbit, and other animals. Recently this work has been repeated and extended by Jowett and Quastel (unpublished) with the help of the tissue slice technique and with the employment of the Warburg manometric apparatus.

The effect of 0.0032 M luminal (phenyl-ethyl-barbituric acid) on the oxidation of glucose by rat brain cortex slices in a phosphate-Locke medium is to bring about an inhibitory action amounting to greater than 50%. There is little or no effect of the drug on the respiration of brain in the absence of glucose, showing that substances are present in brain whose combustion is unaffected by the concentrations of luminal investigated. That the effect of the narcotic at low concentrations is not irreversible is shown simply by washing the brain slices in a Ringer medium after their immersion for an hour at 37° C. in the narcotic solution. The low and steady oxygen uptake found in presence of the narcotic is raised immediately to a higher level which also remains steady. This is shown clearly with such narcotics as luminal, hyoscine, and chlorotone (Quastel and Wheatley, 1934).

* For references to such work, see Voeghtlin *et al.*, 1923; Walker, 1928; Dickens, 1933; Quastel and Wheatley, 1932, *a*; Peters, 1936.

The recovery may be very nearly complete and shows that a truly reversible phenomenon obtains under the experimental conditions employed.

Evipan—N. methylcyclohexenylmethylbarbituric acid—on being injected intravenously, in not too excessive a dose, swiftly causes anaesthesia from which the animal soon recovers. An intravenous injection, bringing about an immediate concentration in the blood of 0.0006 M, causes deep anaesthesia in a rabbit (without the loss of optic reflex) for about ten minutes, after which the animal quickly recovers. Evipan brings about a large inhibition of respiration of brain cortex slices and at a concentration of 0.0006 M the inhibition is of the order of 10%. The inhibition of respiration increases rapidly with the concentration of the drug, the inhibition-concentration curve being sigmoid in shape. It is difficult to be certain of the reality of the shape of this curve at very low concentrations of the drug owing to the possibility that some of the drug may be adsorbed on inert parts of the brain tissue and thus greatly lower its effective concentration. This phenomenon has been noted in investigations on the toxic effects of dyestuffs. Luminal presents similar inhibition-concentration curves to those of evipan with rat or guinea-pig brain. The inhibitive effect of luminal on the oxidation of pyruvate is less than on that of glucose but the typical sigmoid curve is obtained. Again an inhibition of glucose oxidation is encountered at about the narcotic strength of the drug. Although the experimental error in these investigations is of the order 5–10%, it is not unreasonable to conclude from a study of the various inhibition-concentration curves that a real inhibition of glucose oxidation occurs in the presence of brain at the low concentrations of the narcotic which secure narcosis. It is to be emphasized that these results refer to the brain cortex taken as a whole; any large inhibition taking place at very small areas of the brain would not be observed with this technique.

Another phenomenon bearing upon the inhibitory effects of small concentrations of narcotics may now be considered. The steady state of the diminished respiration of brain slices brought about by small concentrations of narcotics such as luminal or chloretone is found to be greatly dependent on the concentrations of potassium ions in the medium. With such high concentrations of K^+ as 0.0128 M—double that normally present in serum—a steady inhibition of respiration is quickly attained by chloretone. At low concentrations of K^+ , e.g., 0.002 M, however, the respiration is found to drop quickly in presence of the narcotics to the level found with the higher K^+ concentrations, to remain at this steady state for a short period, and then to fall quickly, so that eventually the

inhibition found with low K^+ concentrations is very much greater than with the higher K^+ concentrations. The concentration of K^+ normally present in serum is sufficient to steady the inhibition due to the narcotic. The significance of this effect of K^+ is at present obscure; it is highly probable that it is linked with the known effects of K^+ on the respiration of brain cortex slices in presence of glucose. Calcium ions do not appear markedly to affect the phenomenon.

Ether behaves towards the respiration of brain in much the same way as other narcotics, and in general it may be said that its inhibitive effect is directly proportional to the concentration used. The effect of high concentrations of K^+ in steadying the inhibition due to ether is quite as pronounced as in the case of luminal or chloretone, but it is clear that even with high concentrations of K^+ the inhibition of respiration due to ether increases definitely with time and is by no means as steady as with the nonvolatile narcotics. The effect of change of temperature on the inhibition due to ether of guinea-pig brain respiration in presence of glucose is very large. The temperature coefficient between 37° and 42° is about 6.6. As with nonvolatile narcotics, the inhibition due to ether is greatest with glucose. The inhibition of lactate or pyruvate oxidation is less than that of glucose, but there is little or no inhibition of the oxidation of succinate or of α -glycerophosphate.

Turning to organs other than brain, the inhibition of respiration with low concentrations of narcotics is small compared with that of brain. With evipan at a concentration which secures 28% inhibition of the respiration of guinea-pig brain slices in a glucose phosphate medium, no inhibition was found of the respiration of liver and kidney slices in the same medium. Higher concentrations of the narcotics secure inhibitions of respiration. Luminal at a concentration of 0.0032 M brings about a large inhibition of liver respiration and diminishes the increased oxidation due to lactate. It is difficult to state in quantitative terms the inhibitory action of luminal on substrate oxidation by liver or kidney owing to the relatively rapid change of inhibition with time and the large effect of the drug on the respiration of the tissue in absence of added substrate. It is clear, however, that luminal exercises a greater inhibitory effect on the oxidation of pyruvate by kidney and diaphragm than on other substances investigated, and in general it would seem that the effects of low concentrations of narcotics are confined, as with the brain, to the inhibition of the oxidation of substances important in carbohydrate metabolism. With brain, however, in contrast to such tissues as liver or kidney, carbohydrate breakdown seems to be the dominant feature of metabolism, and it is this fact which makes it probable that the specific inhibitory

effects of narcotics are of real significance in the biochemical interpretation of narcosis.

H. R. ING—*Structural Specificity of Drugs*—The structural specificity of drugs is important because it may throw some light on the kinds of chemical or physical processes involved in drug action.

The first type of structural specificity to notice is that due to definite structural units in drug molecules. Here I am concerned with kinds of drug action and not with intensities.

The functional groups of organic chemistry, *e.g.*, OH, NH₂, C:O, C₆H₅, have no intrinsic pharmacological properties, but in many drugs the pharmacological properties can be shown to depend on particular functional groups or combinations of groups. The recognition of such pharmacologically active groups or structural units is the basis of chemotherapy. Such groups have also been shown to occur in some hormones and vitamins. There are three main types of evidence for such groups.

(1) When changes in certain groups of a drug molecule lead to marked diminution or complete disappearance of activity such groups are probably pharmacologically active, *e.g.*, in the cardiac glycosides the unsaturated lactone group appears to be intimately associated with the typical activity, since quite small chemical changes in it, *e.g.*, hydrogenation, lead to inactive or very weakly active compounds.

(2) Conversely, chemical changes that leave the type of activity unaltered distinguish between essential and non-essential structural features. An interesting recent example is the preparation of antirachitic substances by irradiation of sterols with side chains differing from that in ergosterol.

(3) Synthesis of new compounds containing structural features associated with particular pharmacological properties, *e.g.*, chemotherapeutic substances generally, synthetic local anaesthetics, etc.

The existence of pharmacologically active groups in drug molecules is suggestive evidence for a chemical mechanism in their action, especially when such drugs show a highly selective action, *e.g.*, autonomic drugs, cardiac glycosides, hormones, and vitamins. Selectivity of action seems to imply specialized receptors in tissues which respond only to certain structural types.

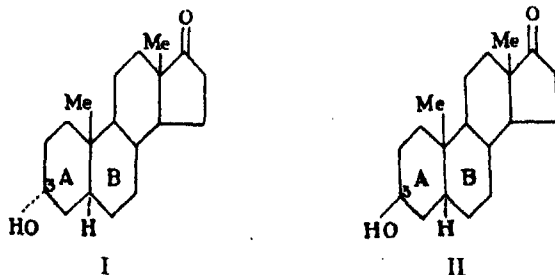
Two difficulties associated with this type of structural specificity may be mentioned. (a) Although definite structural features may be associated with certain pharmacological properties, the same properties may also be shown by drugs of quite different structure. Frequently this may be due to different mechanisms producing the same gross physiological

effects; a gross effect like the death of bacteria may presumably be achieved in a variety of ways and consequently the diversity of chemical antiseptics is not surprising. But it is difficult to account for drugs so unrelated chemically as muscarine and pilocarpine acting selectively at the same sites and eliciting similar physiological responses. (b) Drugs of identical structural type may have entirely different pharmacological actions, *e.g.*, butyl trimethylammonium augments, but its homologue octyl trimethylammonium antagonizes, the action of acetylcholine on the frog's auricle (Raventos, 1936).

Pharmacologically active groups cannot always be demonstrated in drugs, *e.g.*, aliphatic narcotics include a considerable variety of structural types, such as hydrocarbons, alcohols, ethers, urethanes, sulphones, amides, etc. In these cases the drug action must depend primarily on certain physical properties which these classes of compounds have in common.

The effect of structural changes on the intensity of drug action presents many difficulties and as yet inexplicable relations. Only two of the simpler aspects of this subject will be mentioned.

(1) Stereochemical specificity—It is well known that stereoisomeric drugs may have widely different activities. An interesting recent example is provided by the stereoisomerides of androsterone (I) which is 3-*epi*-



hydroxy-aetioallocholanone-17. The two stereoisomerides with the *cis*-configuration of the bridge between rings A and B are inactive. The isomeride with the dihydrocholesterol configuration of the OH group at C₃ (II) has about one-eighth the activity of androsterone (which has the *epi*-configuration at C₃).

This type of specificity is not surprising if the drug or hormonal action involves a chemical combination with receptor molecules in the tissues since *cis-trans* isomerides frequently differ in some of their chemical properties, *e.g.*, in the sterols only those with the dihydrocholesterol configuration at C₃ form digitonides.

The specificity of optical enantiomorphs is particularly interesting because of the identity of their chemical and of all but one of their physical properties. There are many well-established examples of *d*- and *l*-isomerides with widely different activities, *e.g.*, hyoscyamine, adrenaline, ascorbic acid, etc. Cushny (1926), who investigated the subject in detail, argued that a clear differentiation in the activities of optical enantiomorphs indicated that the drug combined chemically with some optically active tissue constituent and that the different activities were to be ascribed to different physical or chemical properties in the compounds so formed. Other factors might also be involved, *e.g.*, different rates of destruction of the optical enantiomorphs by enzymes in the body.

Cushny laid most stress on the different physical properties of the compounds, drug + receptor, but he also recognized that differences in chemical reactivity might be important. Since adsorption may be regarded as chemical combination at a surface, adsorption complexes should also be included as a possibility.

Cushny's views have been criticized recently by Easson and Stedman (1933), who suggest that the different activities of optical isomerides may not be due to molecular dissymmetry as such but to the same causes which produce different activities in symmetrical molecules. They assume that drug molecules combine with asymmetric receptors in tissues and that one enantiomorph "fits" the receptor less adequately than its isomeride. They conclude that it should sometimes be possible to devise a symmetrical molecule which will "fit" the receptor as adequately as the less active enantiomorph and which will consequently have about the same activity. But since it is just the mutual spatial relations of optical enantiomorph and asymmetric receptor in the compound formed which will determine the disparity in physical properties and chemical reactivities of such compounds, it seems unnecessary to introduce the vague notion of "fit".

(2) Homologous series—The relative activities of homologous drugs frequently exhibit a maximum for one member of the series, *e.g.*, *n*-alkyl-phenols and -cresols have maximum phenol coefficients for *n*-amyl members (Coulthard, Marshall, and Pyman, 1930); alkylamines show maximum pressor activity for *n*-hexylamine (Barger and Dale, 1910). Many other examples have been recorded. In such series the pharmacological activity must represent the summation of two opposite effects and depend on at least two physical properties. Unfortunately, a detailed study of the physical properties of such series has not been made.

In some homologous series other factors may come into play. When two active groups are separated by an alkyl chain— $(\text{CH}_2)_n$ —maximum

activity frequently occurs for a certain value of n , *e.g.*, phenylalkylamines, alkylaminoalkyl esters (local anaesthetics and mydriatics), etc. In such examples the distance between the two active groups may determine the ease of combination of the drug with tissue constituents, especially when the combination is a surface reaction (*cf.* Barger, 1930).

There are, however, other examples of maxima in homologous series for which it is difficult to suggest reasons. The relative activities of homologous choline esters (Chang and Gaddum, 1933) show remarkable relations, *e.g.*, the relative potencies per molecule of acetyl-, propionyl-, butyryl-, and valeryl-choline on rabbit intestine are 100, 3, 0.24, and 0.20 respectively; on frog's rectus abdominis the relative potencies are 100, 550, 90, and 25 respectively.

Homologous series are also known in which one member has minimum activity, *e.g.*, in the series $R_4\overset{\oplus}{N}$, where $R = CH_3, C_2H_5, C_3H_7$, and C_4H_9 , minimum curariform activity is shown by $(C_2H_5)_4\overset{\oplus}{N}$, and in the series $RNMe_3$, where R represents the same alkyl groups, $C_2H_5 - \overset{\oplus}{N}Me_3$ has minimum curariform and muscarine-like activity (Ing and Wright, 1933; Raventos, 1936). No explanation has been offered for these examples of minimum activity in a homologous series.

J. H. GADDUM—When the dose of a drug is plotted against the percentage mortality which it produces in a group of animals from the same source an S-shaped curve is obtained. When the variability of the animals is small the curves are approximately symmetrical, but when the variations between individual animals represent a large fraction of the mean lethal dose the curves are markedly skew. These curves could not be symmetrical because no animals are killed by zero, or negative, doses.

If the percentage mortality is plotted against the logarithm of the dose, the curves are always approximately symmetrical whether the variation is large or small. The shape of these curves indicates that if the resistance of an animal is measured by the dose which is just necessary to kill it the logarithm of the resistance is normally distributed. The S-shaped curves are, in fact, integrals of approximately normal frequency distributions. The standard deviation of the distribution is easily calculated from the curve and the slope of the curve is usually expressed in terms of λ , the standard deviation of the normal distribution which most nearly fits the results when the mortality is plotted against the logarithm of the dose to base 10 (Gaddum, 1933).

It has been thought that the use of the logarithm of the dose instead of the dose itself represents a subtle mathematical device for obscuring the peculiarities of some of these curves. This is not so.

Both in practice and in theory the logarithm of any biological measurement is more likely to be normally distributed than the measurement itself. This is well illustrated in the calculations of Hemmingsen (1934) who collected data of linear measurements of the average size of the different species of animals in various phylogenetic groups. The distribution of the logarithms of these measurements was always approximately normal while that of the measurements themselves was often very asymmetrical.

These facts confirm theoretical expectation. The use of the normal formula in studying the distribution of the size of animals may be justified on the ground that it represents the distribution which would be obtained if the size depended on the chance variation of a very large number of factors. If each of these factors produced the same *absolute* effect on large animals as on small, the distribution of the size would be normal. In actual fact each factor probably produces the same *relative* effect on large and small animals. In this case the distribution of the logarithms of the measurement will be normal (Galton, 1879). The common practice of calculating the standard deviation of biological measurements without taking logarithms is justified by its convenience and not by its accuracy.

Similar S-shaped curves are obtained when the dose of a drug is plotted against its effect on an isolated piece of animal tissue (*see* fig. 3). In this case also the use of logarithms gives approximately symmetrical curves. According to Shackell (1923), these concentration-action curves are directly comparable to dose-mortality curves. Instead of a collection of animals there is a collection of cells, which vary in their response to the drug. This theory fits the facts and deserves more consideration than it has received. In the simplest form of the theory, the amount of drug acting on each cell is directly proportional to the concentration in which the cell is immersed. If all the cells responded sharply at the same concentration the concentration-action curve would be a vertical straight line. The variation of the sensitivity of the cells tends to flatten the curve. Since any form of distribution is possible, any curve can be explained in this way. Actually the log-concentration-action curves are approximately symmetrical and the corresponding distributions approximately normal (*see* fig. 4).

We have already been told that skew characteristic curves are found where the concentration-action curve is a hyperbola. If the apparent

hyperbola is due entirely to individual variation, the standard deviation of the logarithm of the susceptibility of individual cells in a given heart (λ) is 0.72. On this theory the heart cells must therefore be very variable

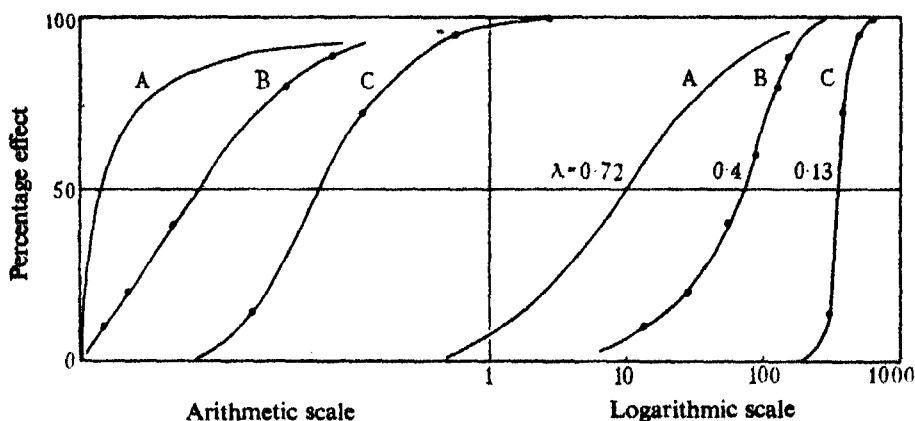


FIG. 3—Effects on isolated tissues plotted against arithmetic and logarithmic scales of concentrations. Data from Clark (1933, *a*). A, hyperbola, acetylcholine, and adrenaline on various tissues (reversible); B, ethyl alcohol on frog's heart (reversible); C, caffeine on frog's rectus abdominis (irreversible); λ = standard deviation of the logarithm of the susceptibility to base 10.

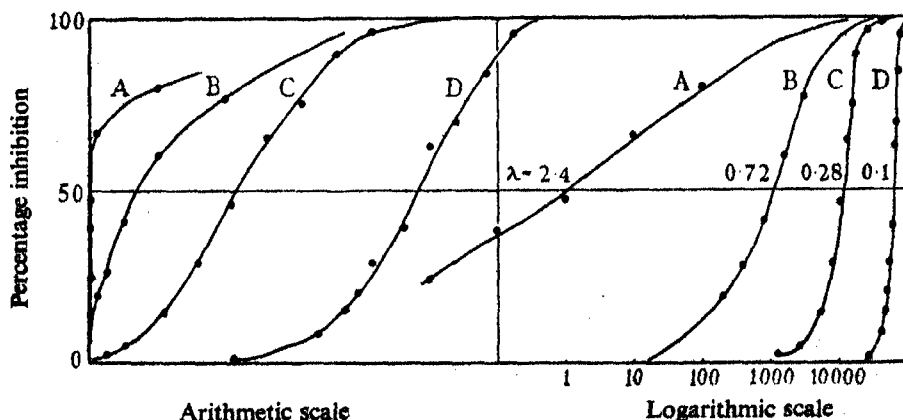


FIG. 4—Percentage inhibition of enzymes by poisons plotted against arithmetic and logarithmic scales of concentrations. A, atoxyl on liver lipase (irreversible) (Rona and Pavlović, 1922); B, quinine on invertase (reversible) (Rona and Bloch, 1921); C and D, nitrophenol on two specimens of invertase (irreversible) (Rona and Bach, 1921).

in their susceptibility. This explains why the characteristic curves showing the variation of the logarithm of susceptibility among different tissues giving hyperbolic concentration-action curves are also flat curves

which become skew when plotted on an arithmetic scale of concentrations.

There are thus two theories current to account for the shape of concentration-action curves. According to Clark (1933), the effect of the drug is directly proportional to the quantity combining with active groups in the tissue and the curve is an expression of the relation between concentration and uptake. Shackell neglects possible complications due to the uptake of the drug not being directly proportional to the concentration, and regards the curves as an expression of the relation between the uptake of the drug and its effect on a mixed population of cells. The two theories are not incompatible, and it is possible that the flatness of concentration-action curves may be due partly to the relation between concentration and uptake and partly to variation of the cells.

Similar curves are obtained when the concentration of a drug is plotted against the inhibition of an enzyme which it causes. When the effect is plotted against the logarithm of the concentration, a series of symmetrical S-shaped curves is obtained, differing only in their slope. Where the poisoning of the enzyme is reversible the concentration-action curve is usually a hyperbola and the results can be accounted for in terms of mass action. It is improbable that variation between molecules of enzyme appreciably affects the shape of this curve. But where the poisoning is irreversible the logarithmic concentration-action curve may be either steeper or flatter than that which corresponds to the hyperbola. These curves may perhaps be explained in terms of variations in susceptibility to the poison of different molecules of enzyme in the solution.

The dissociation curves of oxyhaemoglobin are similar in shape to the other curves discussed. It is possible that their shape is partly determined by continuous variations between the molecules of haemoglobin in a solution.

W. YORKE, F.R.S.—*The Influence of the Host and Parasite on Chemotherapeutic Action*—It has long been known that whilst that form of African sleeping sickness caused by *Trypanosoma gambiense* is curable by various aromatic compounds of arsenic, the form due to *T. rhodesiense* cannot be cured by these drugs. It was therefore with considerable interest that we observed in 1928 that our old laboratory strain of *T. rhodesiense*, which had been preserved by passage through mice since 1923, was just as sensitive to arsenicals as was our old laboratory strain of *T. gambiense*.

Three new strains of *T. rhodesiense* were then obtained from recently diagnosed and untreated Tanganyika cases of sleeping sickness. It was

found that these infections in mice of the early passages could not be cured by arsenicals, but that in mice of later passages they were definitely susceptible to these drugs.

These observations suggested an investigation of the changes experienced by a trypanosome during its maintenance in the laboratory. The trypanosome selected was *T. brucei*, and was obtained in April, 1931, by exposing a dog to the bite of wild *Glossina morsitans* in Uganda; from the infected dog a number of guinea-pigs were inoculated and brought to this country. Since its arrival the parasite has been maintained in three different ways, and the changes undergone by each of these three parallel strains have been carefully observed. The first strain was passaged through guinea-pigs by blood inoculation; the second strain was maintained in a similar manner, except that as often as possible transmission by the tsetse fly replaced blood inoculation; the third strain was passaged through mice by blood inoculation. The characters specially considered were morphology, pathogenicity, susceptibility to arsenicals, and transmissibility by *G. morsitans*.

It was found that all three strains eventually became modified, but that the changes experienced by the mouse-passage strain were much more rapid than those of the strains passaged through guinea-pigs. The mouse-passage strain exhibited changes of morphology; greatly increased pathogenicity for mice and decreased pathogenicity for guinea-pigs; greatly increased sensitiveness to arsenicals; and loss of transmissibility by *G. morsitans*. The guinea-pig-passage strain, which was transmitted from time to time by the tsetse, underwent no appreciable change during a period of four years. Later, when it lost its capacity of infecting the fly, it gradually became more virulent for guinea-pigs and mice and more sensitive to arsenicals.

It follows from this, therefore, that the sensitiveness of the trypanosome to aromatic arsenicals is increased by passage through laboratory animals, but that the rapidity and degree of the increase can be controlled by the manner in which the trypanosome is maintained. It will also be noted that increase in susceptibility to arsenicals is accompanied by an increase in pathogenicity. Passage through mice by blood inoculation results in relatively rapid increase in pathogenicity of the trypanosome for mice and in considerable increase in susceptibility to arsenicals; whilst passage through guinea-pigs by blood inoculation replaced from time to time by tsetse transmission is not accompanied by any appreciable change in either pathogenicity or susceptibility to arsenicals.

Browning and Gulbransen (1935) have also recently drawn attention to the fact that increase in pathogenicity of a trypanosome for mice is

associated with an increase in susceptibility to arsenical treatment. The explanation of this curious paradox is not very obvious, but as a working hypothesis one might postulate that the greater the virulence of a strain of trypanosomes for mice the greater the rate of multiplication of the parasite, and that a trypanosome in a condition of great metabolic activity is more susceptible to the toxic action of a drug than is the same trypanosome when its metabolic activity is on a much lower level.

I will now pass to a brief consideration of another—and from the practical point of view much more important—way in which changes in the trypanosome influence chemotherapeutic action. I refer to the drug-resistance which trypanosomal infections develop as the result of inadequate treatment of the infected animal. This fundamental discovery was made by Franke and Roehl in Ehrlich's laboratory as long ago as 1907, but, although its possible practical significance was at once recognized, knowledge of the subject remained in a chaotic state until 1929, when a technique was elaborated whereby the pathogenic trypanosomes could be kept alive *in vitro* at 37° C. for several days. This technique enabled the subject of drug-resistance to be re-investigated much more advantageously, and the work of the last few years has done much to clarify the subject.

It is now known that the drug-resistance which a trypanosomal infection exhibits after repeated administration of sub-curative doses of a drug is due to a character acquired by the trypanosome itself; it is not necessarily accompanied by any appreciable change in the morphology or pathogenicity of the trypanosome and is not modified when the parasite is transferred to a vertebrate host of a different species. When drug-resistant trypanosomes are exposed to solutions of the drug *in vitro*, it is found that the resistant trypanosomes do not absorb the drug, even when it is present in concentrations rapidly fatal to the normal trypanosomes.

Drug-resistance is only to a limited degree specific. A trypanosome made resistant to one of the aromatic compounds of arsenic is found to be resistant to all the other commonly employed aromatic compounds of arsenic and antimony and also to acriflavine, but not to arsenious acid, tartar emetic, or to Bayer 205. A strain made resistant to acriflavine is exactly similar to one made resistant to an aromatic compound of arsenic or antimony. While it is impossible to make a normal strain of trypanosomes resistant to tartar emetic, a few doses of this drug to an atoxyl- or acriflavine-resistant strain suffice to make it completely resistant to tartar emetic. The production of a Bayer 205-resistant trypanosome

is a long and tedious matter, but such strains can be made, and it is interesting to note that the resistance is specific for Bayer 205.

The ease with which arsenic-resistance is produced during the treatment of experimentally infected animals at once raises the question whether resistant strains are being produced in Tropical Africa, where hundreds of thousands of cases of sleeping sickness are being treated by aromatic arsenicals. The question obviously is of great practical importance from the patient's point of view, because resistance to one aromatic arsenical means resistance to all the commonly employed aromatic compounds of arsenic and antimony. If, moreover, the resistance acquired by a trypanosome should prove permanent and capable of surviving the cyclical passage of the parasite through the insect vector—the tsetse fly—then the problem might well assume an importance of the first magnitude, because a strain made resistant by the inadequate treatment of one patient would be handed on to other individuals by the tsetse fly, and so gradually a condition of affairs would arise in which we were confronted with large numbers of infected human beings who could not be cured by his class of drug.

With the object of elucidating this problem, we have kept various resistant strains under careful observation for prolonged periods. An atoxyl-resistant strain of *T. rhodesiense* has retained unimpaired its original resistance for a period of $7\frac{1}{2}$ years, during which it has been passaged through a series of about 900 mice. Tryparasamide-resistant and acriflavine-resistant strains have likewise shown no loss of resistance after maintenance in mice for $5\frac{1}{2}$ and 3 years, respectively. The Bayer-resistant strain, however, showed a progressive loss of resistance as it was passaged through normal mice. When the resistance was first completely developed in October, 1932, the colossal dose of 10 mg. per 20 gm. of mouse failed to clear the blood of parasites. A year later the resistance had so far decreased that a dose of 0.5 mg. sufficed to clear the blood of a mouse, and 3 years later all trace of resistance had gone, a dose of 0.025 mg. being sufficient to clear the blood of infected mice.

It appears, therefore, that resistance to acriflavine and to the aromatic compounds of arsenic and antimony is rapidly acquired and persists unimpaired for at least $7\frac{1}{2}$ years when the trypanosome is passaged through normal mice by blood inoculation; the resistance to Bayer 205 is, however, slowly acquired and is gradually lost when the trypanosome is passaged in this manner.

In order to ascertain whether a trypanosome retains its acquired character of drug-resistance during its developmental cycle in the invertebrate host—the tsetse fly—we used the freshly isolated strain of *T. brucei*,

to which a reference has already been made. A tryparsamide-resistant branch of this strain was made in September, 1932, by administering sub-curative doses of the drug to an infected guinea-pig. This resistant strain was then maintained in exactly the same way as the parallel normal strain; viz., by passage through guinea-pigs by blood inoculation replaced as often as possible by tsetse fly transmission. In September, 1936—4 years later—when the strain had been passed through a series of 57 guinea-pigs and 4 times through *G. morsitans*, it was found to have preserved its drug-resistance unimpaired. A sub-strain of this tryparsamide-resistant strain was made resistant also to Bayer 205, and this resistance likewise appeared to pass through *G. morsitans*.

This work shows clearly that the acquisition of drug-resistance by a trypanosome does not prevent it from developing in its invertebrate host, and that after its passage through the invertebrate host it still exhibits its acquired character of drug-resistance.

The resistance which trypanosomes acquire to the aromatic arsenicals may therefore prove to be a matter of great practical importance; it is rapidly acquired, persists indefinitely, and survives repeated passage through the tsetse. Resistance to Bayer 205 is unlikely to prove of the same significance, because it is acquired very slowly and is not permanent.

J. F. DANIELLI—The simplest possible concept of the cell surface which is compatible with permeability data, surface tension data, and wetting properties, consists of a lipid layer at least two molecules thick, with a layer of protein molecules adsorbed at each oil-water interface (fig. 5). At the external surface of the membrane there must be an excess of acidic groups, derived from either the lipoids or the adsorbed protein, sufficient for the surface to have a distinctly acid character at biological p_H in the bulk phases. I shall discuss certain of the properties of such a system which are of interest from the point of view of drug action.

1—Since there are acidic ions fixed at the interface, there will be a Donnan equilibrium between the interfaces and the bulk phases, according to the equation.

$$\frac{[Na]_{\text{surface}}}{[Na]_{\text{bulk}}} = \frac{[H]_{\text{surface}}}{[H]_{\text{bulk}}}$$

Owing to the excess of acidic groups at the interface, the surface will in general be more acid than the bulk phases. The maximum possible value of this difference with a cell in Ringer solution or sea-water at p_H 7.5 is two units of p_H . The use of surface-active acids or bases, by changing the p_H at the cell surface, will affect (a) the adsorbed protein

layer, (b) enzyme activity at the surface, (c) the permeability of the lipoid layer.

2—Owing to the oriented dipoles of the oil and protein molecules, at each oil-water phase boundary there will be a potential of the order of 200 millivolts. These potentials will be opposite in direction at the two interfaces, and so will neutralize one another to a first approximation. Whether there is a residual potential, connected with the injury or normal polarization potential, is a point on which there is no evidence. The potential drop at an interface is practically complete over a range of

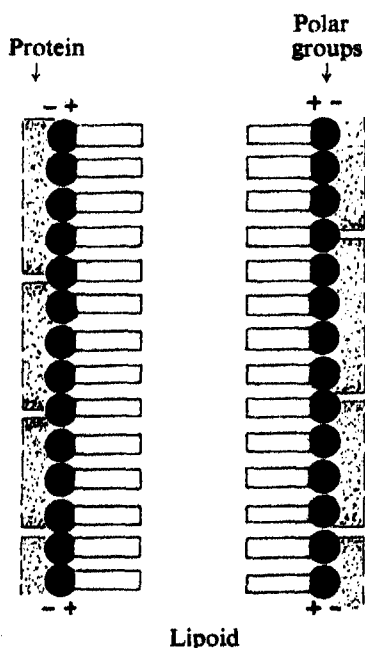


FIG. 5.

5 A. units or less, so the potential gradient at each interface is of the order of 10^7 to 10^9 volts/cm. This potential gradient may be of importance in enzyme reactions at the surface of the cell. Obvious interference with this gradient can be caused by substances adsorbed at either or both interfaces.

3—By measuring temperature coefficients, valuable information can sometimes be obtained about some of the processes involved in a phenomenon. In a one phase system physical processes usually have a low and chemical processes usually, but not invariably, a high temperature coefficient. With cells this simple rule does not necessarily apply, because there are a number of different phases present.

When a substance is entering or leaving a cell it has to pass through the potential barrier at an oil-water interface of the plasma membrane. It cannot pass through such a potential barrier unless it has more than a minimum kinetic energy $mv^2/2$, where v is the velocity component of the ion or molecule perpendicular to the plane of the barrier. The minimum value of $mv^2/2$ is determined by the height of the potential barrier. If it is assumed that the distribution of energy amongst a large number of molecules in solution obeys Maxwell's law, the conclusion can at once be drawn that if the minimum energy for penetration is high, then the temperature coefficient of penetration will be high also, *i.e.*, if a substance penetrates slowly, the temperature coefficient of the rate of penetration will be high. Conversely, if the rate of penetration is high, the temperature coefficient will be low. These conclusions are supported by experimental work on the rate of penetration of inanimate membranes such as zeolite, collodion, and the lipoid-chitin membranes of Crustacea, and of the living plasma membranes of such cells as erythrocytes, marine eggs, and large plant cells.

Hence, at least in those cases where a drug must pass through the plasma membrane of a cell before reaching its site of action, it is not true that a high temperature coefficient implies that the drug enters into a chemical reaction within the cell. It may be that what is measured is the Q_{10} of penetration of the drug into the cell. In order, therefore, to commence the analysis of temperature coefficients, it is necessary to obtain independent measures of the *rate of action* and the *rate of penetration* of the drug.

4—Now consider the plan of the plasma membrane. At each oil-water interface there will be a network of cross-linked polypeptide chains. The sieve-like properties of the plasma membrane may be largely due to this structure. The pore-size of this sieve will be greatly affected by, *e.g.*, surface p_n changes and the salts of heavy metals. In this case drugs may affect the behaviour of the cell without entering or penetrating the lipoid layer of the cell wall.

Finally, one knows that with many cells there are enzyme systems on the cell surface. Are these enzymes scattered at random, or are they arranged in an organized fashion? To investigate this a measuring rod is needed which is capable of dealing with distances of the order of Angstrom units. There is a possibility of obtaining this in the following way. Consider the enzyme centre A, specifically poisoned by the group A'. Suppose that the centres A are distant l apart. Then if one uses a poison of the structure $A' - (R)_n - A'$, *i.e.*, containing two of the poison groups connected by a chain, there will be a sharp increase in efficiency of the

poison when the length of the chain is increased to l or a value greater than l . This increase in efficiency will be due to the fact that both groups of each poison molecule can now react with a centre, whereas, when the chain was shorter than l , only one group could react. If, however, there is no characteristic distance between the enzyme centres A, then there would be no sudden increase in efficiency at any particular chain length. Similarly, to obtain the distance between two dissimilar centres A and B, a poison of the type $A' - (R)_n - B$ could be used. Thus there is at least the possibility of making a map of the enzyme structure of the cell surface.

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*Abstract***The Ultra-Violet Absorption Spectra of Some Complex Aromatic Hydrocarbons**

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Previous work on the ultra-violet absorption spectra of polycyclic aromatic compounds has been extended to include equilin, oestrone, oestradiol, hexahydrophenanthrene, 9:9:10:10-tetramethyl-9:10-dihydro-1:2:5:6-dibenzanthracene, cholanthrene, 6-*isopropyl*-1:2-benzanthracene, fluorene, 1:2- and 3:4-benzfluorenes, 1:2:5:6-dibenzfluorene, 1':2'-naphtha-2:3-fluorene, and 3:4-benzphenanthrene.

The spectra are compared with those previously obtained at room temperature. It was thought of interest to attempt quantitative measurements of absorption spectra of solutions at low temperatures and a technique is described which enables investigations to be carried out down to -120°C . The results for benzene solutions are recorded and it is shown that although in general the maximum extinction coefficient in a given band increases with decreasing temperature, yet in certain bands the reverse is the case. No wave-length shift of the maxima for benzene solutions were observed. Preliminary qualitative experiments on naphthalene, anthracene, and phenanthrene are reported.

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